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13. ABSTRACT *(Maximum 200 Words)*

Amplification of the stimulatory ability of the tumor Ag requires modifications in the tumor Ag.. Previous approaches used replacement of the amino acids in the CTL epitope sequence. Only a few of the resulting CTL showed better recognition of the tumor Ag than the CTL induced by the wild-type epitope. To address this question, we developed a novel approach focused on changes in the side chains of the epitopes. This required first identification of the side chains pointing toward the T-cell receptor. We addressed this question by developing molecular modeling of the peptide HLA-A2 complex. A first point of analysis was Ser 5. We found that removal of the hydroxyl group enhanced the immunogenicity of the tumor Ag (J. Immunol.,. in press). A second point of analysis was the Ala7. We found that when we used unnatural amino acids with linear c-side chains we obtained agonists with the hydrophobic side chain pointing upwards. One of these Ala-7→NLeu (k51) is a significantly stronger stimulator of E75-TCR$^+$ cells than the wild-type E75.

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INTRODUCTION

The overall objective of this project is to develop novel therapeutic approaches to breast cancer by defining the requirements for successful induction of anti-tumor responses using novel immunogens that induce high avidity (hi-av) CTL. Hi-av CTL can overcome the limitations in mediating tumor recognition that are encountered with the currently available low avidity (lo-av) CTL, which are induced by vaccination with specific epitopes of wild-type (w.t.) sequence, delivered as peptides, protein fragments, DNA or RNA. Recently, we developed a novel class of immunogens that can activate HER-2 reactive CTL to recognize the w.t. Ag at a 200-fold lower concentration than vaccine-induced CTL. Their development was based on the hypothesis that an increase in the capability of Ag to form hydrophobic bonds (van der Waals), which stabilize the interacting interfaces should increase their avidity for TCR, leading to full activation compared with weak partial activation by the w.t. Ag. This was achieved by hydrophobic aliphatic side chain extension of the alanine 7 residue of the immunodominant HER-2 CTL epitope E75 (369-377) using linear incremental additions of CH2 (methylene) groups from rare amino acids. These compounds are designated as hydrophobic appendage bearing (HAB) Ag. Their immunogenicity is proportional to the increase in the CH2 side chain length, raising the possibility that the CH2 chain increases signaling by TCR at the Ag-MHC interaction.

We developed a novel approach to address these questions. This is based on the hypotheses that; (a) introduction of the smallest possible changes in the length of the side chain (i.e., methylene, CH2) groups incrementally can have a cumulative effect in increasing he avidity of interaction with TCR by forming hydrophobic bonds; (b) CH2 will not form strong bonds (electrostatic, H-bonds), thus they will be more amenable to induce subtle changes; (c) since TCR has two chains and each reacts with a part of the Ag, progression of these CTL through the stages 1-4.

BODY

During the preliminary phase of the studies supported by this grant, we developed a novel approach for identifying the orientation of the side chains of the CTL epitopes. We modeled the peptide MHC-I structure of the CTL epitope E75 using the pMHC-1 structure of another peptide as a "search model". This first required a search for identification of a peptide which shows the highest structural similarity with E75. As described in the attached manuscript, the HTLV-Tax-1 peptide showed the highest structural similarity to E75. The modeling of the E75-HLA-A2 was initially performed by Mr. Clay Efferson in this laboratory and then by Dr. Ing Maria G. Ioannides. While both Mr. Efferson and Dr. Ioannides could generate model and approximately define the orientation of the side chains, the software available in our laboratory was limited. To speed up the process, we asked for help from Drs. Martin Cheever and Kenneth Grabstein who recommended a distinguished crystallographer, Dr. Darrick Carter. Dr. Carter collaborated with us and generated the energy-minimized /best-fit models of E75-HLA-A2 which are presented in the attached manuscript.

The first models were generated with focus on the central Serine 5 (Ser 5) of the epitope. The models show that the OH group of the Ser 5 points upward toward the TCR. Removal of the OH group leads to variants which will interact with TCR stronger or weaker. The rationale of this hypothesis is that removal of the OH group will either enhance the interaction with TCR or will weaken the interaction with TCR. In the first case the interaction pMHC: TCR will be strengthened by hydrophobic interactions between the methylene group, in the second case the interaction will be strengthened by the interaction with TCR of the OH group. The results in the attached manuscript show that removal of hydroxyl and induction of hydrophobic interactions increased the stimulatory ability of pMHC-1 complexes. Based on the same rationale, extension of the side chain with a charged group will result in an even weaker agonist than the nominal Ag. The results show that activation of human tumor-reactive CTL by weaker agonists than the nominal Ag, followed by expansion with nominal Ag is a novel approach to antitumor CTL development. Fine tuning of activation of tumor reactive CTL by weak agonists, designed by molecular modeling, may circumvent cell death or tolerization induced by tumor Ag, and thus,

may provide a novel approach to the rational design of human cancer vaccines. These studies demonstrated that molecular modeling is a powerful and reliable approach for generation of Ag-variants of higher or lower stimulatory ability than the wild-type Ag. (Please see attached Gallery Proofs of the J. Immunol. paper. **Figure 1** in the paper and the attached prints **(Fig. 1A-D).**

Based on the completion of the studies above, we extended our investigations to the analysis of the positioning of the side chains in the epitope in residues where changes in polarity were not needed. We focused on Alanine 7 (Ala7). The Ala7 model **(Fig 1A)** shows that the CH3 group of Ala7 points side ways. Therefore, we investigated approaches to extend and redirect the side chain upward, toward the TCR. The model with isoleucine 7 (Ile7) replacing Ala shows that a part of the branched side chain of Ile7 points sideways but the smaller branch of the side chain does not **(Fig. 2A,B).** All the substitutions of Ala 7 with unnatural amino acids (valine, leucine) have the same problem.

For these reasons, we decided to break with the tradition and to use unnatural amino acids as replacements for Ala7. The unnatural amino acids γ-amino butyric, Norvaline and Norleucine have linear side chains. When the Ala 7 was replaced with these unnatural amino acids, the models showed a completely different picture **(Fig. 3).** the extension of the C-side chain with CH2 groups lead to gradual upwards orientation of the side chain, which was higher for the NLeu substituted E75. The enlarged area of interest is presented in **Figures 4A,B,C,D.**

E75 with Ala7 substituted with NLeu binds to a significantly higher number of T cells than the wild-type epitope.

To address whether these variants bind T cells we used the newly available HLA-Ig technology. The HLA-A2 dimer can be loaded with variable amounts of peptides and can be used to prepare various pMHC-complexes in the same experiment. We generated dimers loaded with E75 (wild-type) = dE75 and dimers loaded with the NLeu variant (A7.3). Of note, when peptides are synthesized by the synthetic antigen laboratory they are assigned numbers which differ from our

scientific designations. These coded numbers are then used in the laboratory to avoid confusion. This A7.3 is listed in **Figure 3** as K51, A7.2 is listed as K50 and A7.1 is listed as K52.

This test was performed with freshly isolated on ovarian tumor associated dE75 lymphocytes. These lymphocytes accumulate in the abdominal cavity, but are unreactive to the tumor. The results in **Figure 5** show that dK51 bound a significantly higher number of T cells than dE75. There is a seven fold increase in the number of cells than by bound by E75 dimers.

Stimulation with CH2-E75-induced high levels of IFN-γ in weak E75-responding donors.

To establish the ability of CH2-E75 variants to activate T-cells we determined the ability of A7.1, A7.2 and A7.3 to induce IFN-γ in PBMC of two healthy donors, known to develop a weak response to E75 even in the presence of IL-12 (not shown). Each of the A7.1, A7.2, and A7.3-induced higher levels of IFN-γ than E75 in both donors tested. These results suggested that either CH2-E75 stimulations activated a larger number of E75-TCR$^+$ cells than E75, or that CH2-E75 is a stronger inducer of IFN-γ that E75.

To address this question, Donor 1 PBMC were labeled with CFSE and stimulated in parallel with autologous DC pulsed with A7.2 and E75 or as control with DC which were not pulsed with peptide (DC-NP). **Figure 6** shows that, in each division, the number of E75$^+$ TCR, IFN-γ$^+$ cells was significantly higher in the cultures stimulated with DC-A7.2 than in the cultures stimulated with DC-E75. The increase was higher in the cells which underwent three and four divisions compared with cells that either did not divided or divided only one or two times. **Figure 7A** also show that after five divisions (E-H) cells stimulated with DC-A7.2 stained stronger with αIFN-γ mAb (NP=53.9, E75=160.00, K51=224.3) suggesting that they also produced higher levels of IFN-γ. The same pattern of responses was observed during division 5 (**Fig. 7B**). (MCF: NP=56.4, E75=137.5, A7.2=198.9) Thus A7.2-induced both a larger number of T-cells to divide faster than E75 and also higher levels of IFN-γ from each cell.

This work is ongoing. An abstract reporting ongoing work has been submitted to the annual meeting of the Society of Biological Therapy.

REPORTABLE OUTCOMES

1. Characterization of the structure of the peptide bound to HLA-A2 by computer-assisted modeling allows for the first time engineering of the tumor Ag in a rational fashion.

2. The use of unnatural amino acids for construction of tumor Ag allows a controllable approach to immunomodulation using modifications of the van der Waals forces.

3. Development of HAB-variants using CH2 (methylene) appendage as building bricks allowed development of more potent agonists than the tumor Ag. One of these variants Ala7→ Norleucine reacts with a seven fold larger number of T-cells than the wild-type tumor Ag.

4. A first invention disclosure and patent application based on these results has been submitted.

CONCLUSION

1. Molecular modeling of the peptide MHC complex allows identification of the positioning of the side chains and induction of changes in the positioning of the side chains

2. This approach bypasses, at least in part, the requirement for isolation of peptide MHC-1 crystals.

3. Generation of Ag-variants based on C-side chains modification allow modulation of the immunogenicity of the tumor Ag.

4. Priming with weak agonists followed by stimulation with strong agonists is a novel approach to induce, expand, and increase survival of tumor-reactive CTL.

5. Hydrophobic appendage of the C-side chain with CH2 groups leads to variants with modified positions of the C-side chain. This allows binding of more T cells than the wild-type epitope.

6. Unnatural amino acids can be used to generate tumor Ag-variants.

Figure Legends

Figure 1. Molecular modeling of the C-side chain variants of the CTL epitope E75. (Details in the Legend to the Figure 1, attached manuscript. J. Immunol., in press).

Figure 2. Molecular modeling of the CTL epitope E75 with Ala side chain pointing sideways (Fig. 2A) and its substituted variant Ile7 (Fig. 2B). Please note that Ilβ 7 side chain lifts very little towards the TCR.

Figure 3. Molecular modeling of the CTL epitope E75 (A) and of its hydrophobically appended variants; (B) K50 Ala→NVal, (C) K51: Ala7→NLeu, and (D) Ala7→γ -amino butyric. Note the upwards orientation of the NLeu side chain.

Figure 4. Enlarged areas of interest of E75 with (A) Ala7, (B) γ-aminobutyric, (c) Norvaline, (D) Norleucine;

Figure 5. Dimer analysis of $E75^+$ and $K51^+$ cells from ovarian tumor associated lymphocytes (TAL). Control = dNP, OVA-TAL incubated with HLA-A2: IgG dimers not pulsed with peptide; dE75, OVA-TAL isolated with HLA-A2: IgG dimers pulsed with K51.

Figure 6. Percentage of E75 specific IFN-γ secreting cells from PBMC of a healthy donor stimulated with peptides E75 and K51 in dividing cells corresponding to each cycle of division. PBMC were stimulated with K51 but expression of $E75^+$ cells and not of $K51^+$ cells was determined using the same dE75 for both populations. Responding cells were labeled with carloxy fluorescence-diacetate (CFSD).

Figure 7. Dividing cells stimulated with E75 and K51 were gated on the CFSE peak corresponding to division four (7A) and five (7B). Since CFSE labels cells with green fluorescence, cells were stained with HLA-A2:IgG divers labeled with read and with anti-IFN-γ mAb labeled with blue (APC). The gated populations were analyzed for expression of $E75^+$ + IFN-γ cells. $E75^+$ IFN-γ cells are shown in he upper right quadrant (UR).

Publications

Castilleja, A., Carter, D., Efferson, C., Ward, E., Fisk, B., Kudelka, A.P., Gershenson, DM, Murray JL, O'Brian, C.A., and **Ioannides, C.G.** Induction of tumor-reactive CTL by C-side chain variants of the CTL epitope HER-2 (369-377) selected by molecular modeling of the peptide: HLA-A2. *J. Immunology, 169:00-00,2002. In Press.*

Abstracts

Carter, D., Campbell, M., Ward, N.E., O'Brian, C.A., Gershenson, D.M. and **Ioannides, C.G.** Induction of cancer immunity by targeted hydrophobic ladders in the tumor antigen. *The FASEB J.15(4), 946.18, A1200, 2001.*

Ward, N.E., Carter, D., Castelleja, A., Fisk, B., O'Brian, C.A., **Ioannides, C.G.** CTL induced by a variant of the of the HER-2 CTL epitope E75 are resistant to apoptosis mediated by E75. *The FASED J 16(4) A.246.19, 2002.*

Figure 1A

Figure 1B

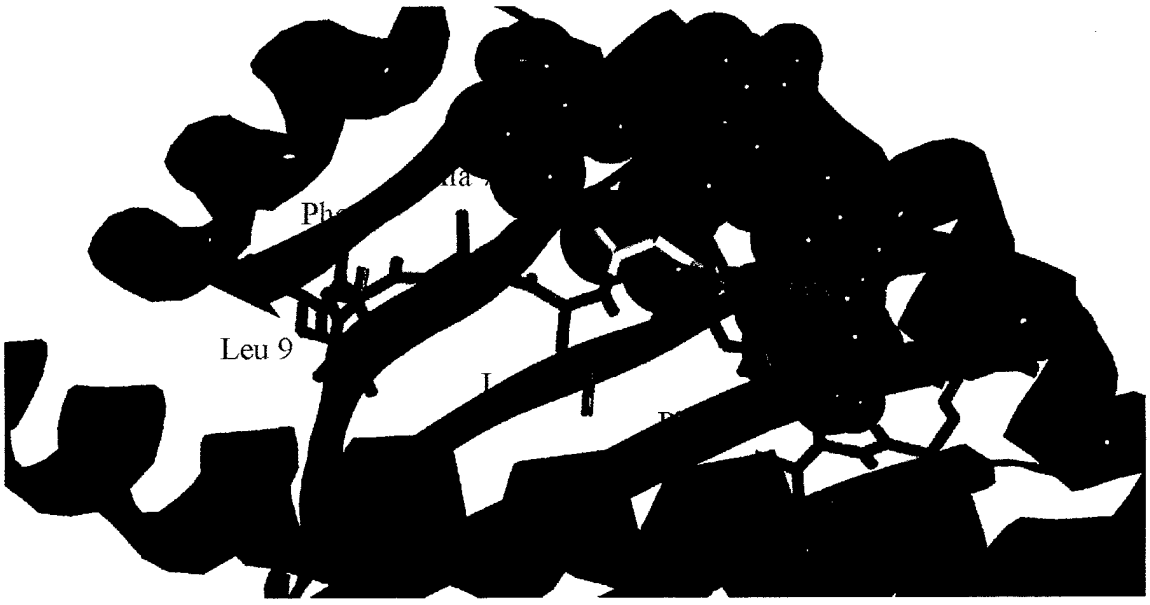


Figure 1C

Figure 1D

Figure 2A

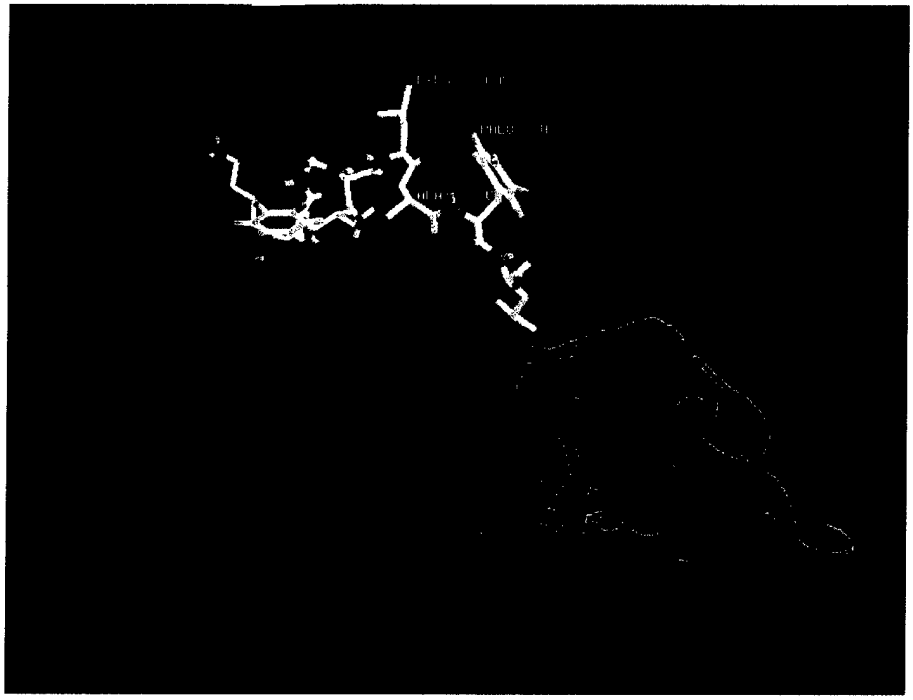


Figure 2B

Figure 3A

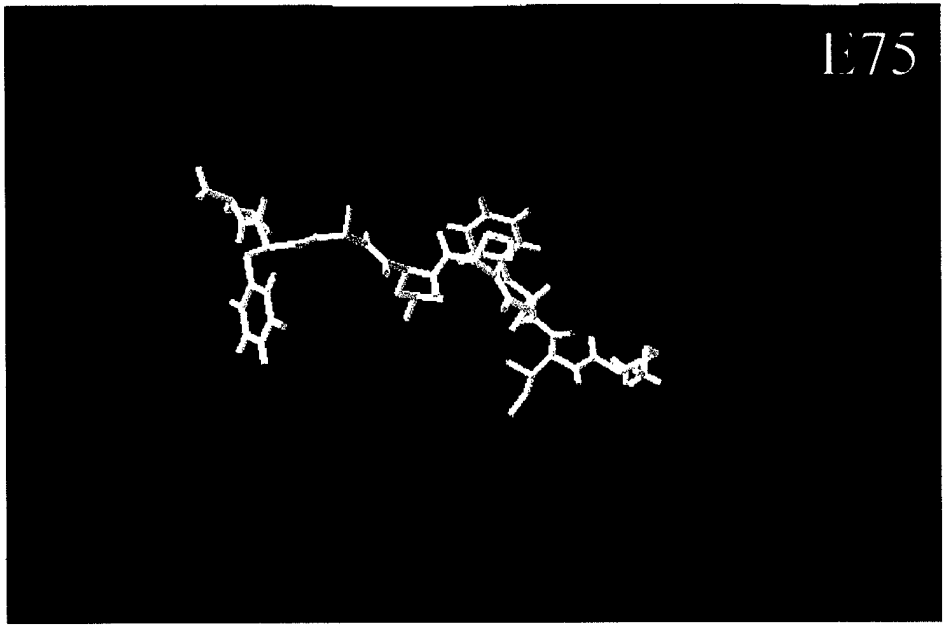
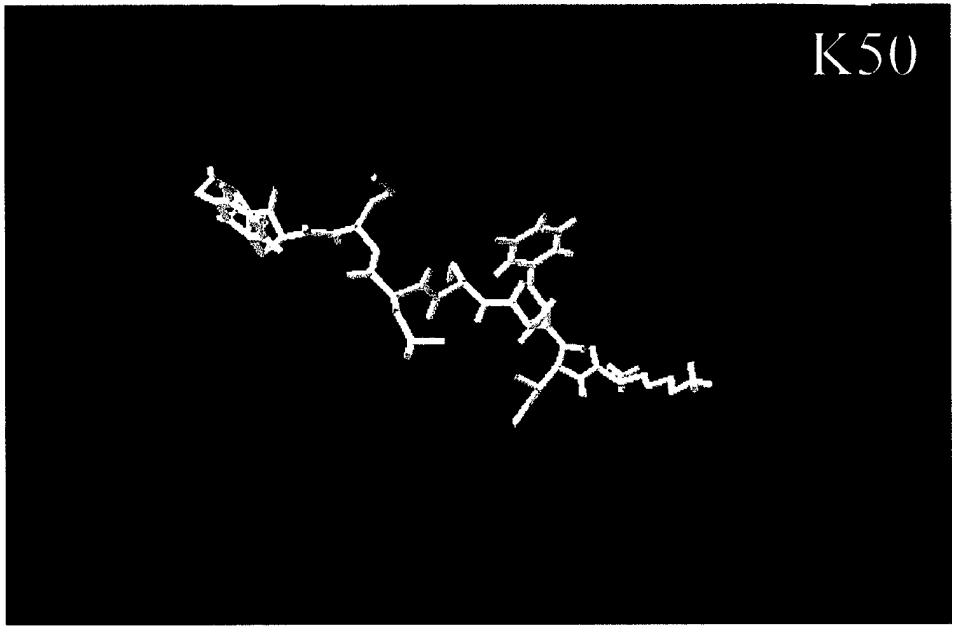


Figure 3 B



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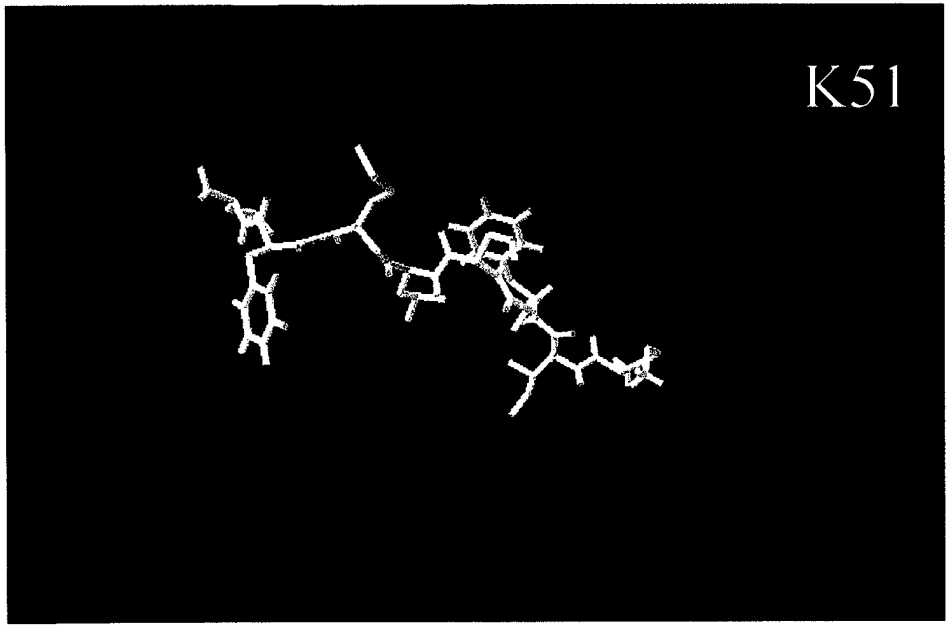
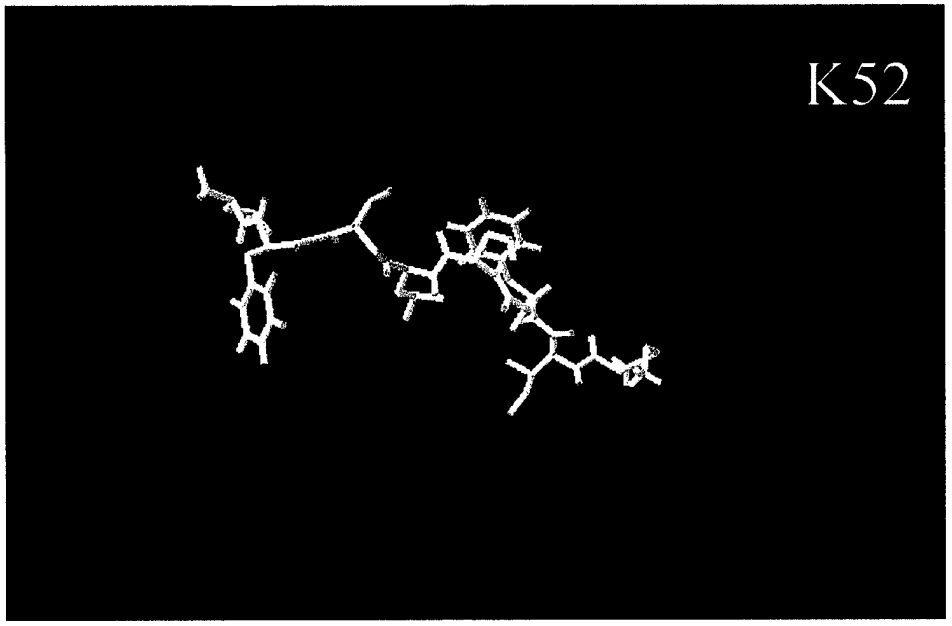
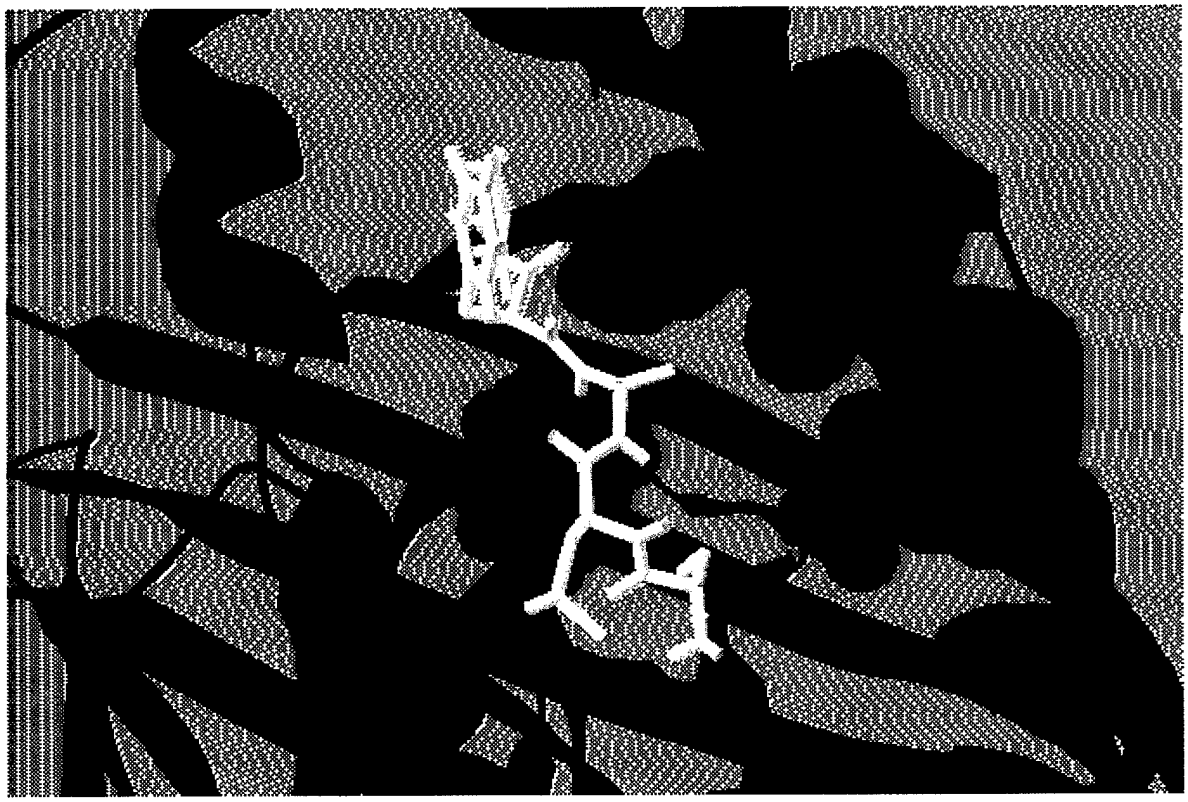


Figure 3D





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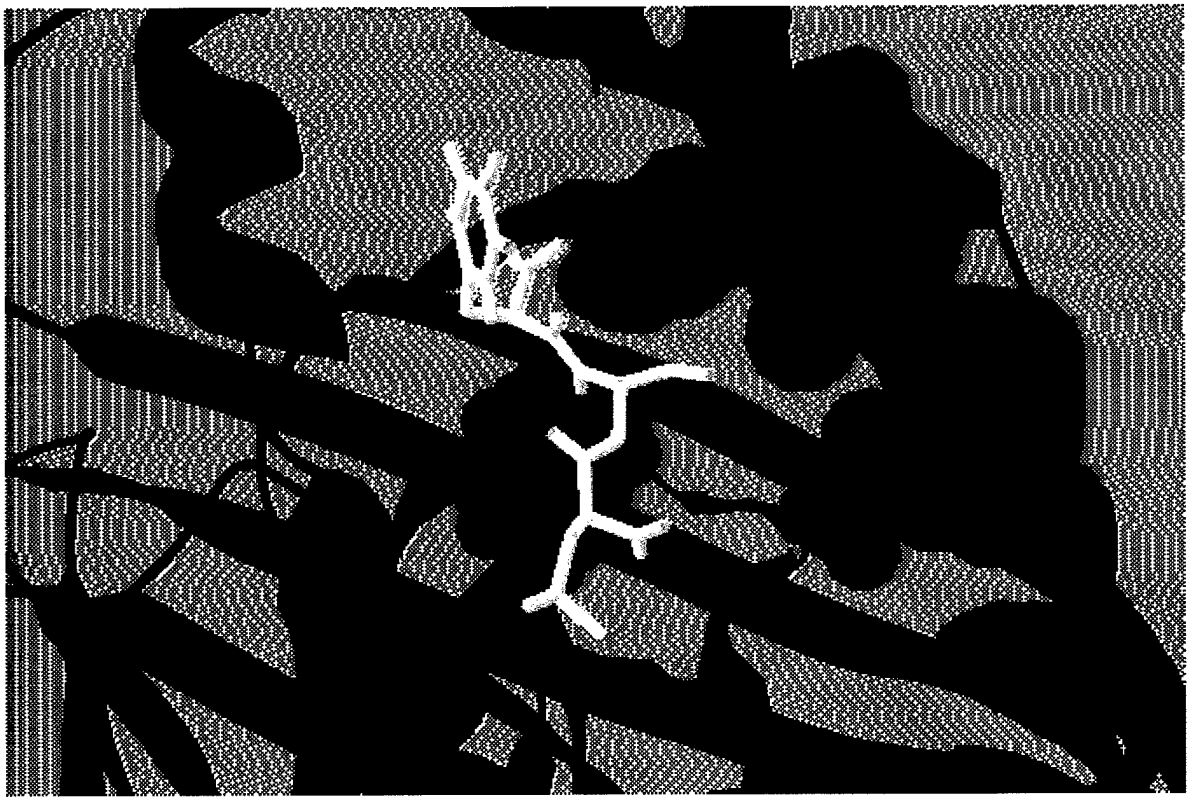

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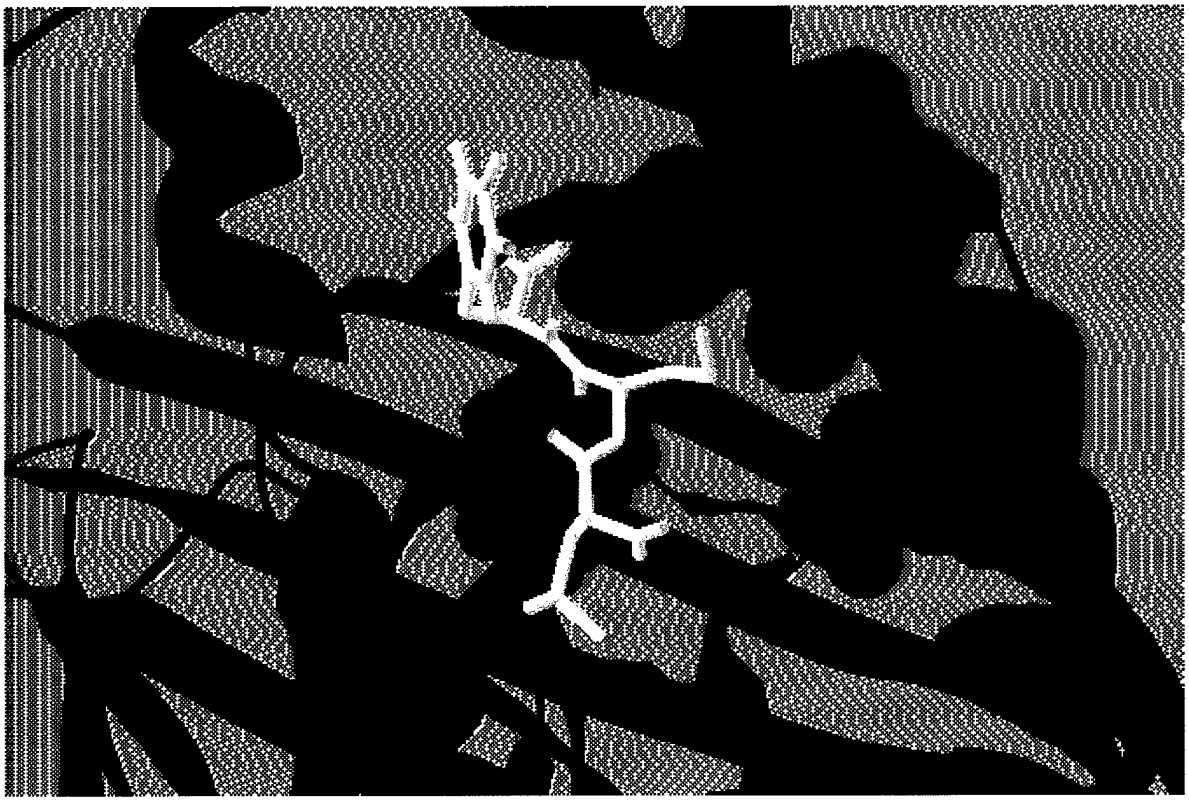

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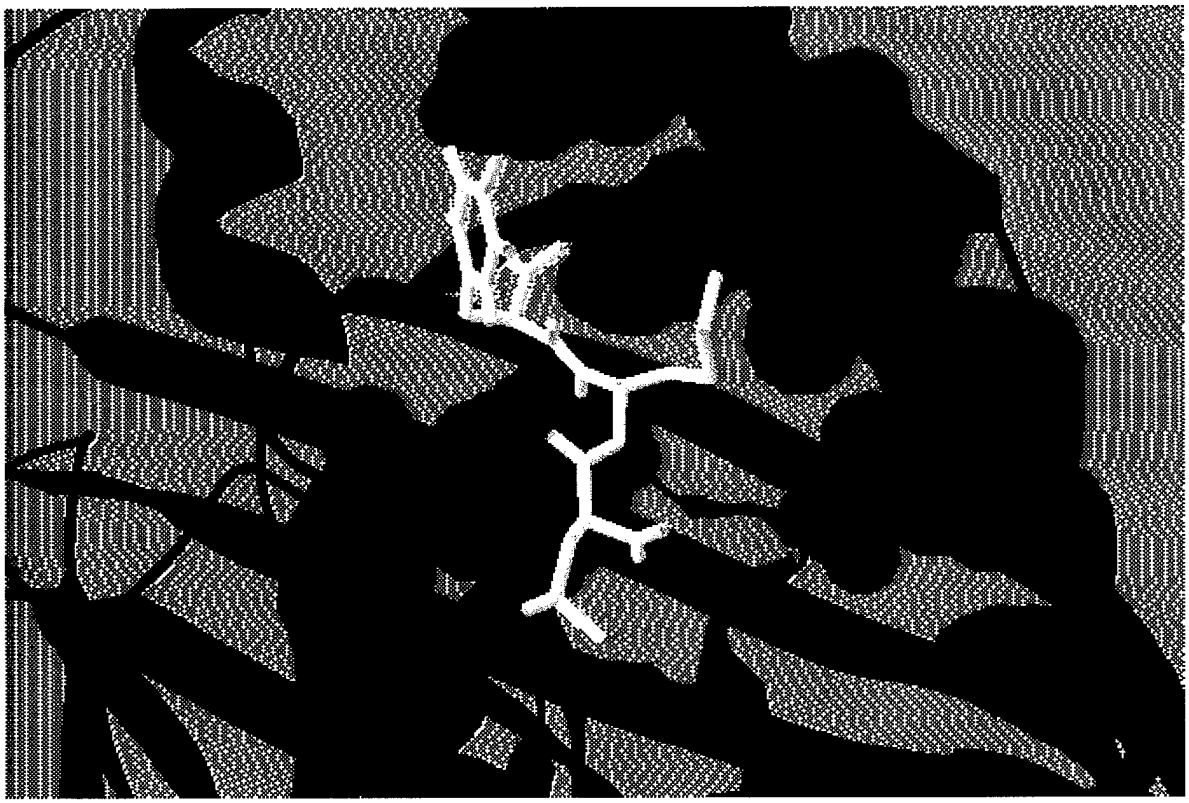
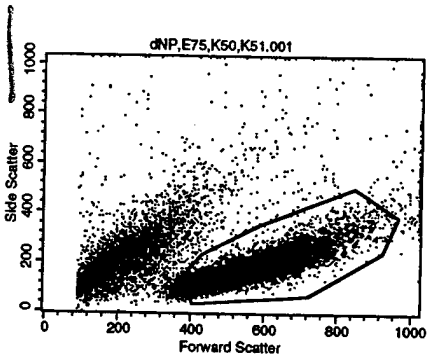
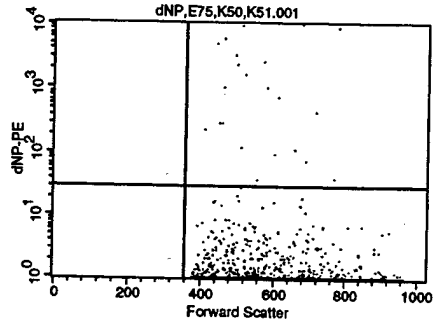


Figure 4D

Figure 5





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Panel:
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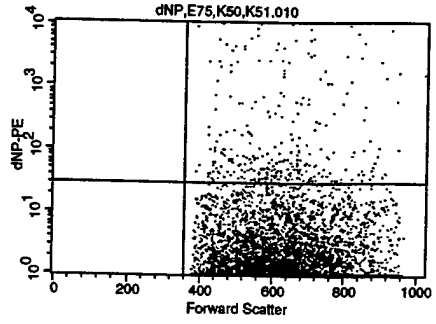
d NP



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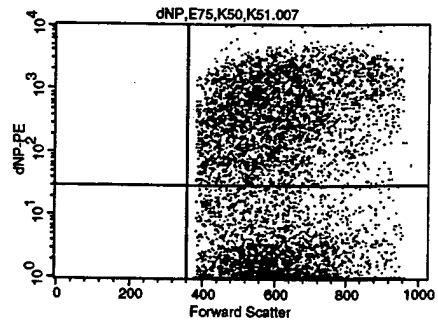
d E75



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d K51

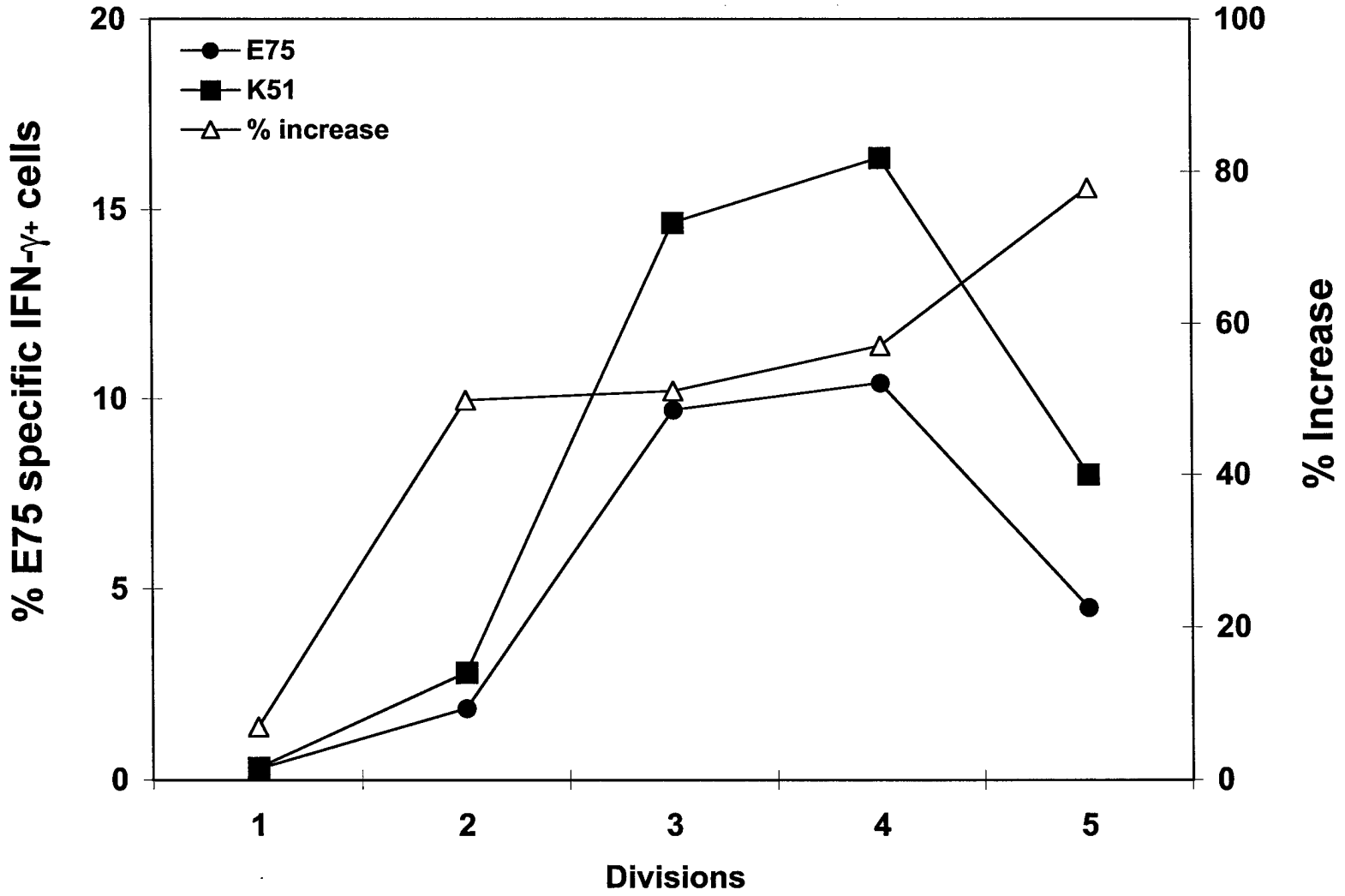
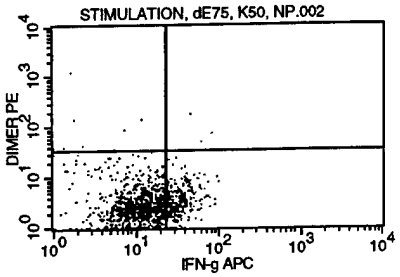

E75
K51
% increase
% E75 specific IFN-γ+ cells
% Increase
Divisions
0
5
10
15
20
0
20
40
60
80
100
1
2
3
4
5

Figure 6



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LL	3887	74.71	2.78	11.49	9.87	3.84	2.68
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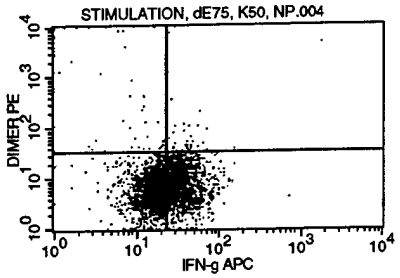
NC



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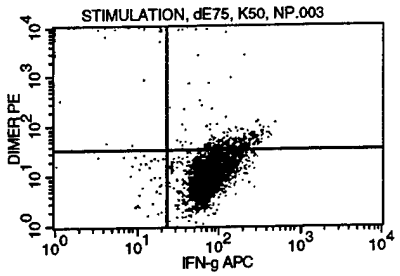
LR	6062	44.54	1.50	38.04	35.35	9.69	7.55

NP



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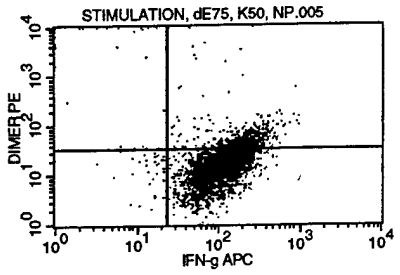
UR	1352	12.01	0.45	159.77	138.88	241.56	57.59
LL	161	1.43	0.05	14.62	13.08	13.24	9.31
LR	9403	83.52	3.13	91.17	83.83	13.37	10.95

E75



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Gate: G1 AND G5
Total Events: 327045

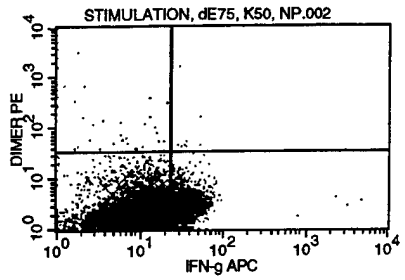
Acquisition Date: 11-Dec-01
Gated Events: 14337
Quad Location: 23, 34

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	281	1.96	0.09	3.85	1.83	8136.06	5493.36
UR	2573	17.95	0.79	224.32	192.64	177.52	56.61
LL	194	1.35	0.06	14.97	13.36	14.32	10.92
LR	11289	78.74	3.45	111.78	98.03	14.48	12.04

K50

division 4

Figure 7A

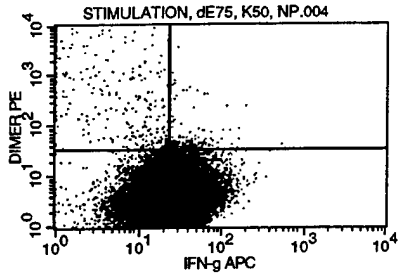


File: STIMULATION, dE75, K50, NP.002
Gate: G1AND G6
Total Events: 139995

Acquisition Date: 11-Dec-01
Gated Events: 55119
Quad Location: 23, 34

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	193	0.35	0.14	6.45	4.05	815.36	183.61
UR	24	0.04	0.02	473.23	51.67	172.87	77.56
LL	43474	78.87	31.05	11.17	9.68	2.42	2.01
LR	11428	20.73	8.16	36.25	33.69	2.96	2.58

NC

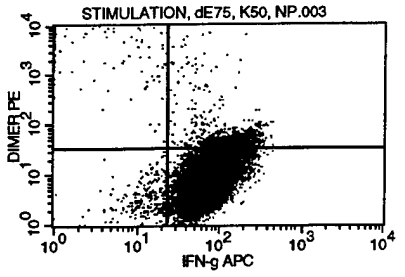


File: STIMULATION, dE75, K50, NP.004
Gate: G1AND G6
Total Events: 403785

Acquisition Date: 11-Dec-01
Gated Events: 155323
Quad Location: 23, 34

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	1440	0.93	0.36	7.60	4.00	2121.02	476.63
UR	748	0.48	0.19	56.35	42.42	138.48	57.02
LL	89506	57.63	22.17	15.86	15.09	5.89	4.69
LR	63629	40.97	15.76	39.15	36.27	7.08	5.41

NP

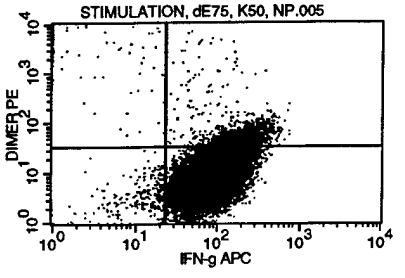


File: STIMULATION, dE75, K50, NP.003
Gate: G1AND G6
Total Events: 300435

Acquisition Date: 11-Dec-01
Gated Events: 116160
Quad Location: 23, 34

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	609	0.52	0.20	5.10	2.36	5084.70	3269.18
UR	5783	4.98	1.92	137.44	123.35	108.89	51.60
LL	981	0.84	0.33	14.74	13.03	4.84	3.49
LR	108787	93.65	36.21	79.67	74.43	10.71	8.54

E75



File: STIMULATION, dE75, K50, NP.005
Gate: G1AND G6
Total Events: 327045

Acquisition Date: 11-Dec-01
Gated Events: 114089
Quad Location: 23, 34

Quad	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
UL	437	0.38	0.13	4.63	2.24	4816.52	2957.24
UR	9679	8.48	2.96	198.86	178.28	94.47	51.79
LL	1125	0.99	0.34	14.89	12.59	4.72	3.46
LR	102848	90.15	31.45	97.58	87.02	11.60	9.26

K50

Division 5

Figure 7B

Induction of Tumor-Reactive CTL by C-Side Chain Variants of the CTL Epitope HER-2/neu Protooncogene (369-377) Selected by Molecular Modeling of the Peptide: HLA-A2 Complex[1]

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To design side chain variants for modulation of immunogenicity, we modeled the complex of the HLA-A2 molecule with an immunodominant peptide, E75, from the HER-2/neu protooncogene protein recognized by CTL. We identified the side chain orientation of E75. We modified E75 at the central Ser[5] (E75 wild-type), which points upward, by removing successively the HO (variant S5A) and the CH2-OH (variant S5G). Replacement of the OH with an aminopropyl (CH2)3-NH3 (variant S5K) maintained a similar upward orientation of the side chain. S5A and S5G were stronger stimulators while S5K was a weaker stimulator than E75 for induction of lytic function, indicating that the OH group and its extension hindered TCR activation. S5K-CTL survived longer than did CTL induced by E75 and the variants S5A and S5G, which became apoptotic after restimulation with the inducer. S5K-CTL also recognized E75 endogenously presented by the tumor by IFN-γ production and specific cytolysis. AQ: A **S5K-CTL expanded at stimulation with E75 or with E75 plus agonistic anti-Fas mAb. Compared with S5K-CTL that had been restimulated with the inducer S5K, S5K-CTL stimulated with wild-type E75 expressed higher levels of E75[+] TCR and BCL-2. Activation of human tumor-reactive CTL by weaker agonists than the nominal Ag, followed by expansion with the nominal Ag, is a novel approach to antitumor CTL development. Fine tuning of activation of tumor-reactive CTL by weak agonists, designed by molecular modeling, may circumvent cell death or tolerization induced by tumor Ag, and thus, may provide a novel approach to the rational design of human cancer vaccines.** *The Journal of Immunology,* **2002, 169: 0000–0000.**

Induction of tumor-reactive CTL by vaccination is a promising approach to cancer therapy. Because tumor Ags are weak immunogens, their immunogenicity must be enhanced if the vaccine is expected to induce antitumor CTL-effector responses. Enhancement of immunogenicity is determined by the ability of the modified agonistic tumor Ag to induce higher levels of effector responses than does the wild-type epitope itself. The higher sensitivity of the agonist-induced CTL for the wild-type Ag is illustrated by higher levels of cytokine secretion and higher levels of cytolysis at the encounter with the tumor Ag or the tumor itself. Strong agonistic immunogens are generally designed by one of two general approaches: 1) to modify immunogens so that they bind the HLA-A, B, C-presenting molecule with higher affinity than their corresponding wild-type counterparts; or 2) to modify the TCR contact site so that agonistic variants of the tumor Ag can enhance the responses of T cells by their TCR contacts (reviewed in Ref. 1). The first approach has been used successfully for normally low-affinity binding HLA-A2 peptides such as MelA, C85, and GP2 (2–7). The second approach is currently used for higher affinity MHC-I-binding peptides. The rationale of the second approach is to replace residues in the Ag that contribute less to the peptide binding affinity for MHC-I and are less likely to contact the TCR with other residues which by their size can create novel contacts for the TCR (1, 8–9).

Mutation of naturally occurring peptides recognized with high affinity at their TCR contacting residues usually results in less potent ligands (10). Thus, mutation of a CTL epitope can lead to a partial agonist or an antagonist. In this regard, one approach for producing stronger agonists has been to modify the surface conformation of the MHC molecule by using buried peptide side chains (11) or buried phenolic groups (12). This also augmented the number of TCR specificities that responded to a single peptide determinant (11, 12). A novel approach to change the MHC affinity for TCR is to modify only the side chains of the amino acids that can contact the TCR. This approach requires identification of such side chains and selective use of modifications so as to enhance tumor Ag stimulation ability while avoiding CTL death from overstimulation. Because only the wild-type Ag is presented in vivo, a central requirement to be fulfilled by side chain modifications of the peptide is that the cells that are activated by the variant must survive at encounter with the wild-type Ag. This means that the wild-type Ag should induce the same or better protection from death by apoptosis in CTL that have been induced by the variant than the variant itself.

Modulation of immunogenicity in this way requires identifying the peptide-MHC-1 complex (pMHC-1) structure, the side chains

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Department of Defense: and G 1141- from the Welch Foundation (C. A. O'B)

pointing upwards in the central peptide area, and using as replacements peptides whose side chains have similar degrees of freedom for flexible orientation at the central position so that they differ in their biological potency. The pMHC-I structure can be modeled by using as a "search model" the crystal structure of another peptide that has structural similarities with the pMHC-I (13, 14). Identification of the positioning of the side chains of the residues in the central area at amino acid positions 4–7 allows changes to be focused in the area that complements the TCR combining site. This area was recently identified as the functional "hot spot" that allows TCR to finely discriminate among similar ligands (10). A side chain in the central area pointing upwards (toward the TCR) can achieve more extensive contact with the TCR than others. This contact is provided by an increase in van der Waals forces from the hydrophobic side chains, or by an increase/decrease in hydrogen bonds by OH groups, or by an increase/decrease in charged interactions. Whether the side chain extension correlates with increased immunogenicity remains unknown.

To address these questions, we examined the binding of the HER-2/neu protooncogene (HER-2), CTL epitope E75 (369-377) to HLA-A2 at the atomic level. Molecular models of the E75-HLA-A2 complex indicated that the side chain of the central Ser^5 (S373) points upward. Thus, the OH group can either enhance binding at the TCR via a hydrogen bond, or sterically hinder the interaction with the TCR by decreasing the affinity of the TCR for the pMHC-I. If the first hypothesis is true, then removal of the OH group should decrease the affinity of binding by the TCR and decrease signaling, hence variants in which the central Ser is replaced by Ala or Gly should be less immunogenic than wild-type E75. If the second hypothesis is true, then Ala/Gly variants should be more immunogenic than the wild-type E75. To address the requirement that variant-induced CTLs survive their encounter with the wild-type Ag, we created another variant reasoning that stimulation with that variant should protect responding cells from death by overstimulation. This variant should stimulate some of the effector functions weaker than E75, and E75 should activate the variant-induced effectors. The only alternatives that would not disturb the peptide bond were positively and negatively charged side chains. Because the negatively charged amino acids Glu and Asp have bulky carboxyl groups, we replaced Ser^5 with the positively charged Lys^5 (variant S5K). The aminopropyl group of Lys extends farther and has a greater flexibility than the acetyl group of the Glu.

Priming with variants S5A and S5G enhanced the induction of IFN-γ and E75-specific cytolysis of CTL from two donors known to respond to E75, but the responders died faster than did the cells that had been stimulated by E75. In contrast, variant S5K induced higher levels of IFN-γ, but not of CTL activity against E75 than the E75-induced CTL (E75-CTL). In a "weak responder" to E75, S5K-induced CTL (S5K-CTL) recognized E75 with lower affinity than did E75-induced CTL. S5K-CTL survived longer than the E75-CTL, which became apoptotic at restimulation with E75. Of interest, restimulation with E75 resulted in better protection from apoptosis in the S5K-CTL than did restimulation with S5K. This protection was paralleled by higher Bcl-x_L to Bad ratios and higher Bcl-2 levels than the ones induced by S5K. Thus, the side chain variants that were less activating than the wild-type Ag induced specific CTL for the E75 expressed on tumors. Such CTL were then expanded by E75, indicating that the nominal Ag or stronger agonistic variants can use priming with weak agonists to bypass induction of apoptosis.

Materials and Methods

Cells, Abs, and cytokines

HLA-$A2^+$ and PBMC were obtained from completely HLA-typed healthy volunteers. T2 cells, ovarian SKOV3, SKOV3.A2 cells, and indicator tumors from ovarian ascites were described (15–17). mAb to CD3, CD4, CD8 (Ortho Diagnostics, Rantory, NJ), CD13 and CD14 (Caltag Laboratories, San Francisco, CA), and HLA-A2 (clone BB7.2; American Type Culture Collection, Manassas, VA) were either unconjugated or conjugated with FITC or PE. Ag expression by dendritic cells (DCs)[4] and T cells was determined by FACS analysis using a flow cytometer (EPICS-Profile Analyzer; Coulter Electronics, Hialeah, FL). GM-CSF of specific activity (1.25×10^7 CFU/250 mg) was from Immunex, Seattle, WA; TNF-α of specific activity (2.5×10^7 U/mg) was from Cetus (Emeryville, CA); IL-4 of specific activity (5×10^6 IU/mg) was from Biosource International (Camarillo, CA); IL-2 of specific activity (18×10^6 IU/mg) was from Cetus; IL-12 of specific activity (5×10^6 U/mg) was a kind gift from Dr. S. Wolf (Department of Immunology, Genetics Institute, Cambridge, MA). The anti-human-Fas mAb CH11 was purchased from Upstate Biotechnology (Lake Placid, NY). mAb to actin, Bcl-2, Bcl-x_L, and Bad were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other specific mAb and isotype controls were obtained from BD PharMingen (San Diego, CA).

Synthetic peptides

Peptides used were E75 (HER-2: 369-377) and its mutated analogs (Table I). To facilitate presentation, E75 variants mutated at Ser^5 are abbreviated based on the position and the substitution. For example, the variant in which serine was replaced by alanine is S5A; the variant in which serine was replaced with glycine is S5G. A7.3 in which the alanine side chain was extended with two methylene groups was obtained by replacement of Ala with Norleucine (linear side chain). F8-1 was obtained by replacing of Phe^8 with isophenylalanine (1 CH2) deletion. All peptides were prepared by the Synthetic Antigen Laboratory of M. D. Anderson Cancer Center (Houston, TX) and purified by HPLC. The purity of the peptides ranged from 95–97%. Peptides were dissolved in PBS and stored frozen at −20°C in aliquots of 2 mg/ml.

Molecular modeling of the peptide: HLA-A2 complex

The coordinates of the native HLA-A2 structure (14, 18, 19) were downloaded from the Brookhaven protein database (ID number: 3HLA). This file was used as a template for manipulations with the Swiss Model (20) program available through the Expasy web site. The Tax peptide bound to the HLA-A2 (21) was mutated manually to yield the bound E75 peptide and the Ala^5, Gly^5, and Lys^5 variants. Each new structure was submitted for energy minimization with the GROMOS96 implementation of the Swiss-PdbViewer. Solvent-accessible surface area was calculated with the GETAREA1.1 online program with the default probe radius, set at 1.4 Å.

T cell stimulation by peptide-pulsed DC

DCs generated from peripheral blood were plated at 1.2×10^5 cell/well in 24-well culture plates and pulsed with peptides at 50 μg/ml in serum-free medium for 2 h before the addition of responders, as described (15, 16). E75-induced and S5K-induced CTL lines were maintained by periodic stimulation with peptide pulsed on DCs, followed by expansion in the presence of irradiated feeder cells and PHA. The number of cells expressing a TCR that was specific for HLA-A2 bound to the E75 peptide (E75-TCR^+ cells) was performed using E75 dimers (dE75) prepared as described in the manufacturer's instructions. Empty HLA-A2:IgG dimers were obtained from BD Pharmingen. Control without peptide dimers not pulsed with peptide (NP) were prepared in parallel and tested in the same experiment. Positive control influenza matrix peptide M1 (58–66) dimers (dM1) were prepared simultaneously and used in the same experiment. For analysis, cells were incubated in parallel with dNP, and dE75 followed by PE-conjugated anti-mouse IgG1. Intracellular expression of Bcl-2 was determined, following manufacturer's instructions using FITC-conjugated Bcl-2, Ab, and a matched FITC-conjugated isotype control.

CTL and cytokine assays

Recognition by CTL of peptides used as immunogens was performed as described (17). Recognition of E75 and of its variants was considered specific when the percent specific lysis of T2 cells pulsed with E75 minus

[4] Abbreviations used in this paper: pMHC-I, peptide-MHC-I complex; HER-2, HER-2/neu protooncogene; DC, dendritic cell; NP, not pulsed with peptide; FW, forward scatter; d, dimer.

Table I. *HLA-A2 binding stability by E75 and its variants*[a]

Code	Sequence	Binding Stability	Ligation[b] Strength	Change
E75	KIFGSLAFL	482	28	Wild type
K1G	GIFGSLAFL	138	28	Positive charge→neutral
S5A	KIFGALAFL	482	28	OH→nonpolar aliphatic
S5G	KIFGGLAFL	483	30	OH→neutral
S5K	KIFGKLAFL	482	29	OH→positive charge
F8K	KIFSGSLAKL	88	30	Aromatic to (+) charged
F8Y	KIFGSLAYL	482	28	OH in aromatic residue
F8D	KIFGSLADL	236	28	Aromatic to (−) charged
A7.3	K1FGSL (NLeu)FL	nd[c]	nd	2 CH2 extension of Ala[7]
F8-1	K1FGSLA (Iso-Phe)L	nd	nd	1 CH2 deletion of Phe[8]

[a] The binding stability is an estimate of half time of dissociation (in minutes) from HLA-A2 of peptides of the sequence listed above. The theoretical half-life of dissociation was calculated using Parker's algorithm (27) available at http://bimas.dcrt.ig.gov/molbiol/hla-bind.

[b] The ligation strength was calculated using the SYFPEITHI program (28). The experimentally determined mean channel fluorescence values for HLA-A2 expression on T2 cells after incubation with peptides and staining with MA.2.1 mAb were: NP = 90, E75 = 305, S5G = 295, S5A = 290, S5K = 285, K1G = 240, and F8Y = 305.

[c] nd, not done.

the SD was higher by at least 5% than the percentage of specific lysis of T2 cells that had been pulsed with peptide plus the SD, as described (22). A significant increase/decrease in CTL activity was defined as an increase/decrease of >20% in the lysis of T2 cells pulsed with peptide by variant-induced CTL compared with wild-type E75-induced CTL. Similarly, a significant increase in IFN-γ induction was defined as an increase of >20% in IFN-γ levels after stimulation with the variant vs after stimulation with the wild-type E75. The 20% value was chosen as a cut-off for significant increase based on the assumption that if a 2-fold increase of the minimum 5% increase (defined above) is 10%, then an increase >10% should be significant if it equals at least 20%. Equal numbers of viable effectors were used in all assays. IL-2, IL-4, and IFN-γ were detected using cytokine ELISA kits (Biosource International or R&D Systems, Minneapolis, MN) with a sensitivity of 4–7 pg/ml (15).

Apoptosis assays

E75- and S5K-CTL lines were activated by autologous DCs pulsed with various concentrations of E75 or S5K in the presence or absence of 100 μg/ml of CH11. For anti-CD3-mediated apoptosis, OKT3 mAb was absorbed on wells of 96-well plates overnight before addition of lymphocytes (23). For day 1 apoptosis assays, IL-2 was not added to the cultures. For day 4 apoptosis assays, IL-2 (300 IU/ml) was added to the cultures at 24 and 72 h after stimulation with DC-pulsed peptides. Detection of Fas-mediated apoptosis was performed in the presence or absence of the agonistic mAb CH11 (anti-Fas mAb) as described (23). Cells were labeled by incubation in PBS containing 0.1% Triton X-100 and 50 μg/ml propidium iodide, and the DNA content was determined by using flow cytometry.

Western analysis

A total of 2×10^6 S5K-CD8$^+$ cells were stimulated for 96 h with E75, S5K, A7.3, or F8-1 peptides pulsed on DCs at a final concentration of 25 μg/ml. Additional controls included cells that were stimulated with T2 that had not been pulsed with peptide, or S5K cells that were not stimulated or cells that were stimulated with PHA. A total of 20 μg of protein from supernatants from 10,000 g of postnuclear detergent lysates were separated on a 12% SDS-PAGE gel and immunoblotted as described (24). Membranes were probed with monoclonal anti-actin, anti-Bcl-2 (1:500), anti-Bad (1:500), or anti-Bcl-x_L (1:500) in 1% BSA-TBS containing 0.1% Tween 20 for 2 h at 25°C, and probed with peroxidase-linked sheep anti-mouse Ig (1:1000) in 1% BSA-TBS containing 0.1% Tween 20. Immunoreactive bands were detected by ECL as described (24).

Results

Generation of E75 variants directed by molecular modeling

The rationale for this approach was to identify amino acids in E75 permissive to replacement that would be substituted without abolishing the objects of the variant peptide to induce CTL responses. Substitutions in side chains that maintain the overall conformation of the peptide backbone in the HLA-2 are more likely to lead to cross-reactive Ag for wild-type Ag-specific CTL than are substitutions that change the peptide backbone conformation. We modeled the E75-HLA-A2 complex by replacing the human T cell leukemia virus-1 peptide Tax with E75. The Tax peptide (25, 26) shows the highest structural similarity with E75 of the models available in the databases. The Tax sequence *LLFGYPVYV* is similar to that of E75:*KIFG SL AFL* with respect to the position of aromatic residues in P3 and P8 and the aliphatic side chain extensions in the first four and the last three amino acids (only K1 and F8 differ by an NH3 and an OH group extension). The major differences rest in the central area P5 P6:YP vs SL. One Tax analog, P6A, shows even more similarity with E75 YA vs SL, with Ala and Leu differing only in the propyl side chain. This comparison allowed identification of the side chains that point upwards or sideways and will be more likely to contact TCR. The results show that the side chains of Lys[1], Ser[5], and Phe[8] point out of the binding pocket of the MHC (Fig. 1*A*). The side chains of Phe[3], Leu[6], and Ala[7] point toward the helical "walls" of the pocket (Fig. 1*A*). The models of the TCR-pMHC-I (HLA-A2) interaction predict that of the side chains pointing away from the MHC, Ser[5], Leu[6], and Ala[7] are most likely to contact the CDR3 (Vα + Vβ) region. We focused on Ser[5] because the change induced by the removal of the hydroxyl group was likely to have the strongest effects.

Ser was substituted with Ala, Gly, and Lys. These substitutions removed an HO-group (Ala), a HO-CH2-group (Gly), or replaced the OH group with the aminopropyl (CH2-CH2-CH2-NH3) group. The position of the OH suggests that it is less involved in interactions with the HLA-A2 (Fig. 1*A*). No significant changes of the MHC molecule were necessary to accommodate these modifications (Fig. 1, *B–D*). Ser[5] is preceded by Gly[4], which because it does not have a side chain, is very flexible and may allow small accommodations in the model. The positions of Phe[3] and Lys[1] that precede the Ser[5] seem to be unchanged among the four models. These results indicate that Ser[5] is in a good structural position to allow side chain replacements in the antigenic peptide that can modify its interactions with TCR. S5A, S5G, and S5K bound to HLA-A2 with similar affinity as did E75 (Table I). In T2-stabilization assays, S5A, S5G, and S5K showed similar stabilizing ability for HLA-A2 as determined with mAb MA2.1 (Table I, legend), and similar scores for times of dissociation and ligation strengths (Table I) with those of E75 as determined using the HLA-peptide binding prediction (27) and SYFPEITHI programs (28).

Increased IFN-γ-inducing and E75-specific CTL-inducing ability of the E75-variants S5A and S5G

To address whether modification of the E75 side chain by deletion or extension would increase or decrease the ability of the modified Ag to stimulate CTL induction and survival, we tested several healthy donors known from previous studies to produce E75-specific CTL at priming ("strong responders", donors 1 and 2) or exhibit weak CTL activity after several repeated stimulations (weak responders, donor 3). PBMC were stimulated in parallel with autologous DCs pulsed with E75 variants. Donor 1 responded with higher levels of IFN-γ at priming with variants S5K, S5G, and S5A, and lower levels of IFN-γ at priming with control variants F8Y and F8K than at priming with E75 (Fig. 2, *A* and *B*). CTL induced by priming with E75 recognized E75 better than did CTL induced by S5K, F8Y, or F8K, whereas CTL induced by S5G and S5A recognized E75 better than CTL induced by E75. S5A and S5G induced both higher levels of IFN-γ and higher cytolytic activity than did E75. Thus, removal of the OH group correlated with

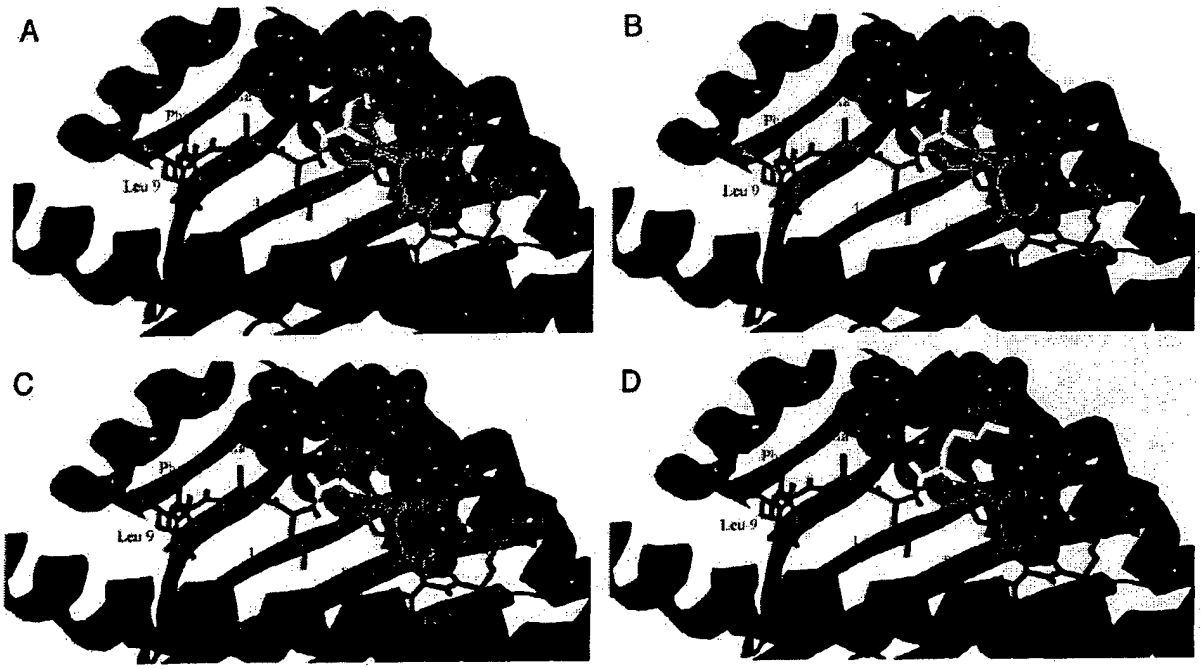

FIGURE 1. *A*, Molecular model of the E75 peptide bound to the MHC. The MHC-peptide complex was modeled as described in *Materials and Methods*, and rendered using the POV-Ray implementation of the Swiss PdbViewer. Residues within 6 A of Ser[5] are displayed with their van der Waals radii in red. Ser[5] is in yellow, while the other E75 peptide residues are in green. The model is presented rotated with an angle of 180° to facilitate distinction of the side chains pointing upwards. *Top*, ~~anti~~-2 domain; *bottom*, ~~anti~~-1 domain of HLA-A2. HLA-2 is depicted in blue. *B*, Similar view of the complex for the Ser[5] to Ala[5] model of the peptide-MHC complex. *C*, The Ser[5] to Gly[5] model. *D*, The Ser to Lys[5] model. The same orientation is used as in all the molecules above.

alpha alpha

higher IFN-γ induction and higher lytic activity against E75. CTL induced by S5K secreted higher levels of IFN-γ, but their recognition of E75 was weaker. Thus, replacement of OH group with aminopropyl group had more selective effect than removal of the OH group. Extension of these results with cells from donor 2 revealed that all at the E75 variants induced higher levels of IFN-γ at priming than did E75: S5K by 36%, S5A by 100%, and S5G by 64% (Fig. 2*C*). Significantly higher levels of IFN-γ were detected 96 h after stimulation with each variant in response to the highest dose (25 μg) of exogenously pulsed peptide in the presence of IL-2 for 2 days. Significant differences in IFN-γ induction were not observed when E75 or its variants were used at 1.0 or 5.0 μg/ml at 48 or 72 h. The E75-specific lytic activity of CTL induced by S5A was significantly higher than the lytic activity of CTL induced by E75 (Fig. 2*D*). The increase in lytic activity by S5A paralleled the increase in IFN-γ in response to S5A. Recognition of E75 by S5K-CTL was lower than the recognition by E75-CTL. CTL induced by the E75, S5K-CTL, and S5A-CTL all recognized the indicator SKOV3.A2 tumor. To determine whether E75-specific tumor-lytic CTLs were present in the variant-induced CTL, we performed cold-target inhibition of tumor lysis. Tumor lysis by S5K-CTL was inhibited less by T2-E75 than lysis by E75-CTL (Fig. 2*E*). This confirmation that S5A can induce both higher IFN-γ and higher lytic activity against E75 suggested that the OH group of Ser[5] hindered the TCR interaction with peptide-HLA-A2 and that removal of the OH group allowed a stronger TCR activation. However, at restimulation, the number of cells stimulated by S5A and S5G dropped faster than the number of cells that had been stimulated by E75. Cells stimulated by S5K survived longer than E75-stimulated cells (Fig. 2*F*), suggesting that the stimulus from the (CH2)3-NH3 was more effective than stimuli from the CH3 or the CH2-OH in maintaining the survival of responders.

Stimulation with S5K enhanced survival of responding T cells

Cancer patients are weak responders to E75 and require repeated stimulation for CTL induction. To clarify the differences between E75 and S5K in the induction of cytolysis, we tested T cells from donor 3 for whom several stimulations with E75 were required to induce detectable CTL activity, but responded with IFN-γ secretion at priming (16). S5K and E75 induced similar levels of IFN-γ at priming and at restimulation (Fig. 3*A*). The kinetics of induction F3 of E75-specific CTL in relation to the number of stimulations is shown in Fig. 3*B*. E75 again induced higher E75-specific lytic activity than did S5K. Like donor 2, E75-stimulated cells from donor 3 declined in number after the third stimulation with Ag more than the S5K-stimulated cells (Fig. 3*C*). These results showed that S5K induced better survival in responders than E75. These results were confirmed in subsequent stimulation experiments. In parallel experiments, priming with E75 induced lower levels of Bcl-2 in $CD8^+$ cells than did priming with S5K. There were only small differences in Fas ligand, Fas, and IL-2Rα ex- AQ: C pression between E75-stimulated and S5K-stimulated donor 3 $CD8^+$ cells (A. Castilleja et al., unpublished observations). AQ: D

and C. G. Ioannides

S5K-induced CTL recognized E75 with lower affinity than E75-induced CTL

Weaker recognition of E75 by the S5K-CTL raised the question of whether S5K induced smaller numbers of CTL than E75, or whether the CTL induced by S5K had lower affinity for E75 than for S5K. To address the recognition of variant-induced CTL, we tested their ability to recognize E75 and the inducing variant in parallel. S5A-CTL (donor 1) recognized S5K weaker than S5A (24% decrease), suggesting that extension of the CH2 side chain in

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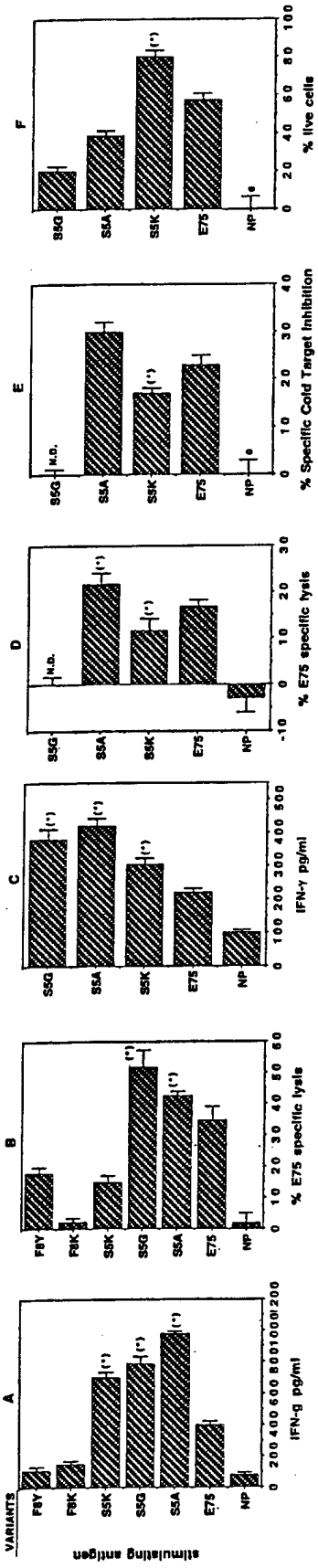

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position 5 with OH and (CH2)3-NH3 groups, respectively, hindered TCR recognition. Similarly, donor 3 S5K-CTL recognized E75 weaker than they recognized S5K (Fig. 4*A*). To verify that S5K is recognized with lower affinity than E75 by donor 3 E75-CTL, we performed concentration-dependent lysis. E75-CTL recognized S5K with lower affinity than E75. S5K recognition was close to recognition of E75 (32 vs 41%) only at high concentrations (50 μg/ml; Fig. 4*B*). Similarly, S5K-CTL recognized E75 with lower affinity than S5K (Fig. 4*C*). These results demonstrated that the OH and aminopropyl groups selectively modulated the affinity of recognition. To address whether E75-specific CTL were present in smaller numbers in S5K-CTL, we tested recognition of E75 at the same concentration (10 μg/ml) at four E:T ratios (10, 20, 30, 40). Even at the highest E:T ratio of 40:1, S5K-CTL recognized E75 (25.4% lysis) to a significantly lesser extent than did E75-CTL at an E:T ratio of 10:1 (48.2% lysis).

S5K-CTL recognize endogenously presented E75

Because S5K-CTL survived longer than E75-CTL, this raised the possibility that S5K could be used to induce CTL-recognizing tumors. To determine whether S5K-CTL recognized endogenous E75 in cytolysis assays, we performed cold-target inhibition of tumor lysis. T2-E75 inhibited lysis of freshly isolated ovarian tumor OVA-16 (HLA-A2$^+$, HER-2^{high}) by 21% in an 8-h CTL assay, and by 45% in a 16-h assay (Fig. 5, *A* and *B*). Similar inhibition (38%) was observed against SKOV3.A2 in a 16-h assay (data not shown). These results indicated that S5K-CTL recognized the endogenously presented E75 and ovarian tumors overexpressing HER-2. The levels of inhibition of lysis indicative of specific recognition were similar to those levels observed with donor 2, E75-CTL, and S5K-CTL (Fig. 2*E*). We also tested S5K-CTL ability to secrete IFN-γ at an encounter with the ovarian tumor SKOV3.A2 and its HLA-A2$^-$ counterpart SKOV3. This was necessary because the tumor and responding lymphocytes shared HLA-A3. S5K cells secreted high levels of IFN-γ within 20 h, when IL-12 was used as costimulator (Fig. 5*C*). IFN-γ was induced even in the absence of IL-12, but at lower levels. mAb inhibition experiments indicated that IFN-γ secretion was associated with recognition of HLA-A2. (data not shown). This indicated that present among the S5K-induced CTL was a subpopulation of cells that recognized endogenously presented E75 by cytolysis and IFN-γ secretion.

FIGURE 2. Induction of effector functions in donor 1 (*A* and *B*) and donor 2 (*C–E*) at priming with the wild-type CTL epitope E75 and its variants. *A* and *C*, IFN-γ; *B*, *D*, and *E*, Cytolysis. *A* and *C*, IFN-γ was determined from supernatants collected from the same cultures which were used on day 8 for CTL assays. *B*, *D*, and *E*, Equal numbers of effectors from each culture were tested in the same experiment. Results indicate the percentage of E75-specific lysis obtained by subtracting the specific lysis of T2 cells not pulsed with peptide, from the specific lysis of T2 cells pulsed with 25 μg/ml E75 in the same experiment. The E:T was 20:1. Stimulators were autologous DCs pulsed with 25 μg/ml peptide. NPs indicate control effectors that were stimulated only with autologous DCs which were not pulsed with peptide. *E*, Effectors E75-CTL, S5K-CTL, and S5A-CTL lysed the indicator ovarian tumor SKOV3.A2. Specific cold target inhibition indicates the percentage of inhibition of lysis of SKOV3.2 cells by cold (unlabeled) T2-E75 cells minus inhibition of lysis in the presence of T2-NP cells. S5G-CTL were not used in this experiment because their numbers declined rapidly after restimulation. E:T ratio was 30:1, cold:hot ratio was 10:1. *F*, Percentage of live cells in donor 2 cultures primed and restimulated with each variant 30 days after priming. Note the decrease in live cells in cultures stimulated with S5A or S5G. *, $p < 0.05$.

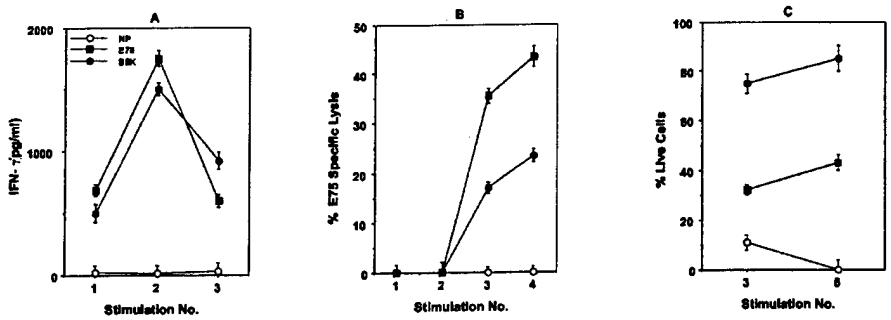

FIGURE 3. *A*, Kinetics of IFN-γ production; *B*, E75-specific CTL induction; and *C*, survival of donor 3 CTL stimulated by E75 and S5K. Experimental details as described in the text and the legend to the Fig. 2. *A*, IFN-γ was determined on day 3 after stimulation with each peptide. The numbers 1, 2, and 3 indicate the number of stimulations. Equal numbers of live cells from E75- and S5K-stimulated cultures were stimulated with autologous DC pulsed with the corresponding peptide. *C*, The number of live cells recovered was determined 1 wk after the third and the fifth stimulations.

Antiapoptotic effects of E75 in S5K-activated CD8⁺ cells

Induction of CTL by the variant S5K raised the question of whether such cells could survive an encounter with E75 since E75 is present in vivo. To address whether E75 can induce CD95-mediated apoptosis, E75-CTL and S5K-CTL were stimulated with E75 and S5K in parallel in the presence of the agonistic Ab CH11. Three days after stimulation with E75, 46% of the E75-CTL had undergone apoptosis, whereas only 15.4% of the S5K-CTL were apoptotic after stimulation with S5K (data not shown). In contrast, when S5K-CTL were stimulated with S5K or E75, cells stimulated with E75 survived longer and may have increased in number as compared with the cells stimulated with S5K. Stimulation of S5K-CTL with 25 or 50 μg/ml E75 for 4 days increased the number of CD8⁺ cells by 26 and 64%, respectively. Stimulation of the same cells with ~~S5K~~ anti-Fas increased their numbers by 0.93 and 27%, respectively (Fig. 6, *A* and *B*), but no increase in cell number was observed in the absence of CH11. Notably, S5K-CTL continued to respond to S5K with higher levels of IFN-γ, but lower levels of IL-2, than did cells treated with E75 (data not shown).

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To address whether E75 and S5K interfered with apoptosis pathways, S5K-CTL were restimulated with E75 or S5K at two different concentrations or remained unstimulated (Group 0, DC only) in the presence of CH11. Apoptosis analysis was performed at 24 and 96 h. Both E75 and S5K inhibited the residual Fas-apoptosis within 24 h and this inhibition was peptide concentration-dependent (Fig. 6*C*). When apoptotic cells were counted on day 4, both peptides were protective, but E75 seemed to be more protective than S5K (Fig. 6*C*, day 4).

To confirm the antiapoptotic effects of E75 and S5K on S5K-CTL, we performed cell cycle analysis. Analysis of cells in the subG$_1$ phase (Fig. 6*D*) showed that 46% of the unstimulated S5K cells became apoptotic. E75 and E75 + CH11 inhibited this apoptosis by 83%. S5K had a slightly lower inhibitory effect (63% inhibition). S5K + CH11 reduced apoptosis by only 24% compared with unstimulated S5K-CTL confirming the results in Fig. 6*B*. The percentage of cells in G$_1$ phase (resting) was similar in both stimulated and control unstimulated cells (50 ± 5%). The percentages of CD8⁺ cells in S phase in cultures stimulated with E75 or S5K were also similar. Of interest, the proportion of cells in the S phase was higher in cultures stimulated by E75 + CH11 than in cultures stimulated with S5K + CH11, suggesting that E75 transmitted a stronger stimulatory signal for division of S5K-CTL than their original inducing Ag. The differences between cells in the G$_{2/M}$ phase were small compared with the unstimulated cells, and they were not considered significant. These results agree with the higher proliferation of S5K-activated CD8⁺ cells in response to E75 than to S5K (Fig. 6, *A* and *B*).

Apoptosis resistance in stimulated T cells at day 4 is mainly due to the intrinsic pathway (29). Because resistance to Fas-induced apoptosis was suggestive of TCR-induced protection, we investigated the effects of E75 and S5K in up-regulation of Bcl-2, Bcl-x$_L$, and Bad. Unstimulated and DC-NP-stimulated CD8⁺ cells from

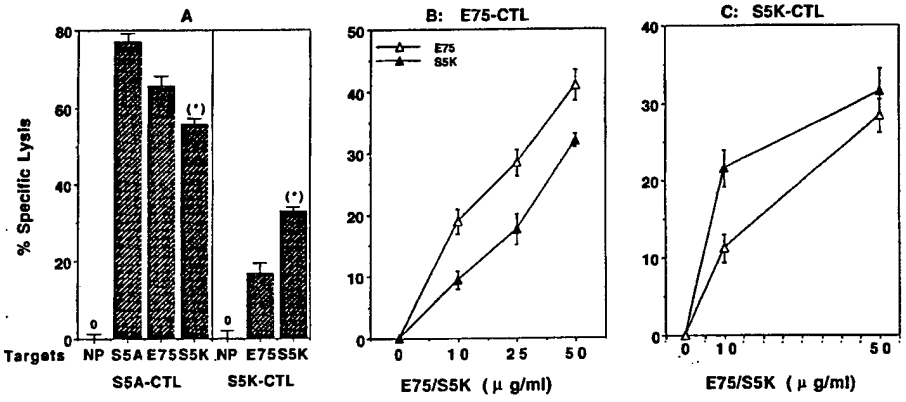

FIGURE 4. Ag specificity of S5A-CTL, S5K-CTL, and E75-CTL. *A*, Donor 1 S5A-CTL recognized S5K less efficiently than S5A. Donor 3 S5K-CTL recognized E75 with lower affinity than S5K. T2 cells were pulsed with E75 and S5K at 10 μg/ml. *B*, Donor 3 E75-CTL recognized S5K with lower affinity than E75. *C*, Donor 3 S5K-CTL recognized E75 with lower affinity than S5K-CTL. Concentration dependent recognition of E75 and S5K in the same experiment. Targets were T2 cells pulsed with the indicated concentrations of peptide. *B* and *C*, Results of a 6-h CTL assay. E:T ratio was 10:1. *, $p < 0.05$.

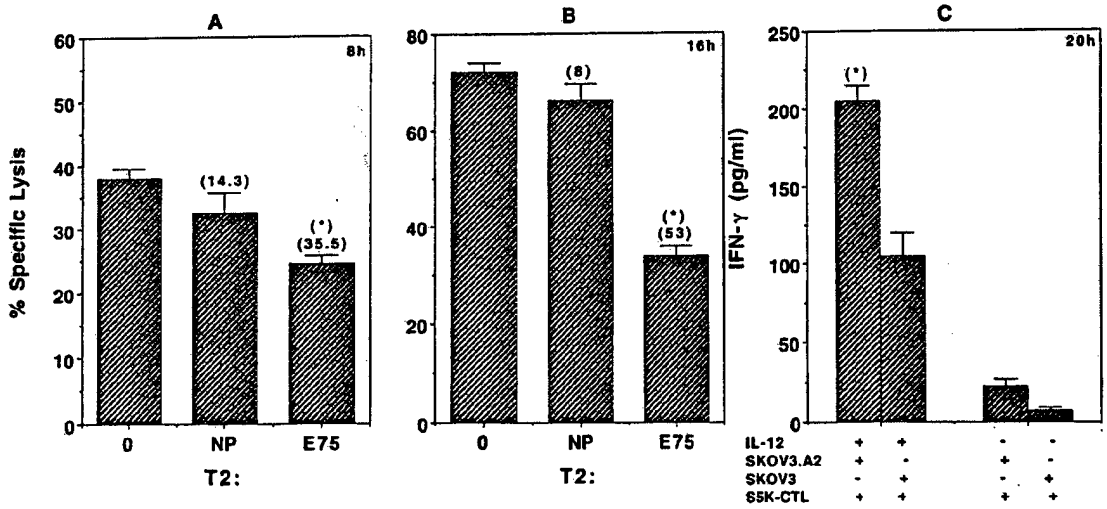

FIGURE 5. S5K-CTL recognized endogenous E75 presented by ovarian tumor cells. *A* and *B*, Cold target inhibition of cytolysis of OVA-16 (HLA-A2, HER-2^{high}). Cold targets were T2 pulsed with E75, using as specificity control T2 which were not pulsed with peptide (T2-NP). Numbers in the parentheses indicate the percentage of inhibition of lysis of S5K-CTL by T2-E75 compared with lysis of tumor in the presence of T2-NP. *, $p < 0.05$. E:T ratio was 10:1; the ratio of cold to hot targets was 1:1. *C*, IFN-γ induction. IL-12 was used at 3 IU (300 pg/ml); the responders to SKOV3. A2 stimulator ratio was 40:1.

S5K-CTL were used as negative controls, while S5K-CTL stimulated with the agonists A7.3 and F8-1 were used as positive controls. E75 induced a higher Bcl-x_L to Bad ratio than S5K. A7.3 and F8-1 variants induced even higher Bcl-x_L to Bad ratios than E75, indicating that their effects were sequence-specific (Fig. 7*A*). S5K was a slightly stronger up-regulator of Bcl-2 than E75. The inhibitory effects of E75 and S5K on Bad up-regulation were similar, although E75 was a slightly stronger inhibitor. These results indicate that E75-mediated protection from CD95-mediated apoptosis of S5K-CTL correlated with down-regulation of proapoptotic family members. The increase in the level of expression of Bcl-2 was considered significant compared with the up-regulation of Bcl-2 induced by a mitogen (PHA) in the same cells for 96 h. This is evident when the Bcl-2 and Bcl-x_L to actin ratios are compared at stimulation with S5K and PHA vs the Bcl-2 and Bcl-x_L to actin ratios in unstimulated cells (Fig. 7*B*). For S5K stimulation, the ratios are 1.72 (Bcl-2) and 1.32 (Bcl-x_L), while for PHA stimulation the ratios are 1.55 (Bcl-2) and 4.37 (Bcl-x_L). The increase in the levels of Bcl-2 and Bcl-x_L at stimulation with PHA is comparable with the increase reported in other studies in the presence of a mitogen, but in the absence of IL-2. Increase in the Bcl-2 levels is in general observed if mitogen-activated T cells are given high doses of IL-2 (30, 31). Thus, activation and expansion of tumor-reactive CTL by the variant S5K allowed better survival of these CTL in response to the wild-type tumor Ag.

To address whether E75 and S5K stimulation affected expansion, TCR expression, and Bcl-2 expression in E75$^+$TCR cells, S5K-CTL were stimulated with T2 cells pulsed with either E75 or S5K or not pulsed with peptide (T2-NP). The number of E75$^+$TCR cells was determined. One week later, to determine whether the affinity of the TCR for E75 was affected by the stimulation, we assessed expression of E75$^+$TCR cells both immediately

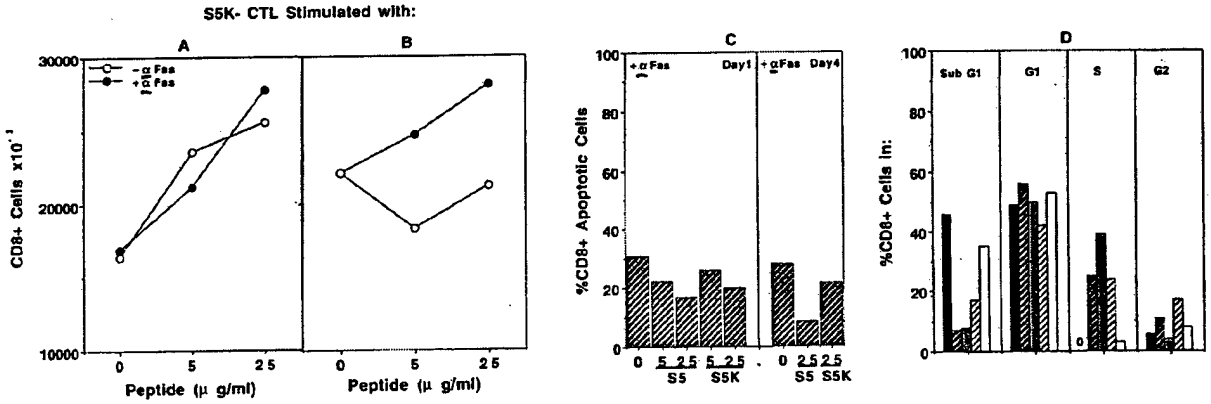

FIGURE 6. Expansion of CD8$^+$ cells from S5K-CTL after stimulation with E75 (*A*) or S5K (*B*) in the absence (○) or presence (●) of CH11 mAb. Equal numbers of S5K-CTL were stimulated with DCs pulsed with 0, 25, and 50 μg/ml of each peptide. The number of CD8$^+$ cells was determined by flow-cytometry using anti-CD8 mAb-FITC conjugated. *C*, Ag-induced resistance to CD95-mediated apoptosis. S5K-CTL were stimulated with autologous DCs pulsed with E75 or S5K at 5 and 25 μg/ml or control no peptide (0). CH11 mAb was added 1 h later. The number of apoptotic cells was determined 1 and 4 days later. *D*, Restimulation with E75 and S5K-induced resistance to CD95-mediated apoptosis in S5K-CTL stimulated 1 wk before with S5K. Apoptotic cells are shown in the panel subG1. Results are from one experiment representative of three independently performed experiments. Bars indicate unstimulated (■), E75 stimulated (▨), E75 + anti-Fas stimulated (▩), S5K-stimulated (▨), and S5K$^+$ anti-Fas stimulated (□).

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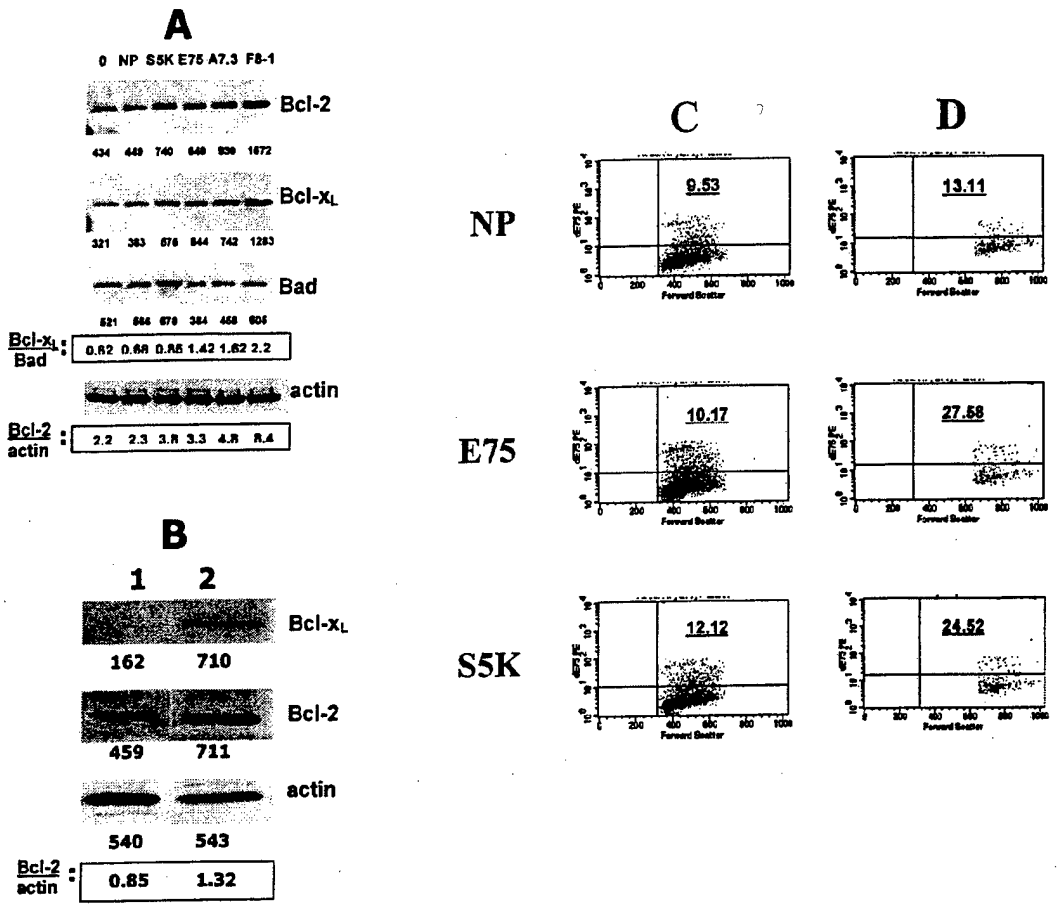

FIGURE 7. *A*, Expression levels of Bcl-family members by S5K-CTL stimulated with the indicated peptides; or *B*, with PHA for 96 h. The same blot was used for probing with all Abs. 1 indicates unstimulated; 2 indicates PHA-stimulated cells. The numbers below the bands indicate the densitometric values (pixel total $\times 10^{-3}$) *C* and *D*, Expansion of E75$^+$TCR cells in S5K-CTL stimulated in parallel with T2-E75 (E75), T2-S5K (S5K), or with T2-NP (NP) as control for 1 wk. The presence of E75$^+$TCR cells was determined using dE75 (*y*-axis). Forward scatter (FW) is shown on *x*-axis. *C*, E75$^+$TCR cells expression in large lymphocytes (FW: 640-1000); *D*, E75$^+$TCR expression on small lymphocytes (FW: 380–600). The percentage of dNP$^+$ cells ranged from 0.1–0.5% in both populations.

after staining and after an additional 50-min incubation of dE75-
F8 stained cells in PBS (Fig. 7, *C* and *D*, and Fig. 8*A*). For further refinement, E75$^+$TCR expression and Bcl-2 expression were analyzed separately in two gated populations of smaller size (FW scatter: 380–600) and of larger size (FW scatter: 640-1000). In the small lymphocytes (Fig. 7*C*), the percentages of E75$^+$TCR cells were similar in all three stimulation groups and the E75 and S5K-stimulated S5K-CTL appeared to have similar affinities for dE75, which were stable >50 min. In contrast, in the larger lymphocytes, the percentage of E75$^+$TCR cells was higher in the E75-stimulated than in S5K-stimulated S5K cells (Fig. 7*D*). The affinity for E75 also seemed to be higher in the E75-stimulated group than in the S5K-stimulated group (Fig. 8*A*). Because E75-stimulated cells proliferated better than S5K-stimulated cells, we calculated the number of E75$^+$-TCR cells in each stimulated culture. The number of E75$^+$-TCR cells in both small and large lymphocytes stimulated by E75 was higher than in the S5K-stimulated S5K-CTL (Fig. 8*B*). The percentage increase was similar to the increase observed in CD8$^+$ cells (Fig. 6, *A* and *B*). This finding confirmed that S5K-induced CTL expanded better when restimulated with E75 than when restimulated with S5K. The levels of E75$^+$TCR and Bcl-2 in the E75-stimulated S5K-CTL in the large lymphocytes were also higher than in the S5K-stimulated S5K-CTL (Fig. 8, *C* and *D*). This suggested that stimulation of S5K-CTL with E75 resulted in changes in receptor distribution or conformation that increased the binding of dE75 as suggested by Braciale and Spencer (32). These effects were not observed in the small E75$^+$TCR lymphocytes. Bcl-2 levels were higher in the small lymphocytes after stimulation with S5K compared with E75. E75-stimulated S5K-CTL recognized E75 both as peptide and when endogenously presented by tumor (data not shown). Together these results indicate that priming CD8$^+$ cells with agonists for induction of cytolysis that are weaker than the nominal wild-type Ag followed by restimulation with the wild-type Ag can bypass induction of apoptosis either by the wild-type Ag (at priming) or by the weak agonist (at restimulation). This effect leads to increased survival and expansion of antitumor effectors.

Discussion

In this paper, we investigated the possibility of using molecular models of peptide:HLA-A2 complexes to select side chains that can induce CTL responses in T cells recognizing the HER-2 CTL epitope E75. The E75-HLA-A2 model identified several residues, Lys^1, Ser^5, Phe^8, that point upwards and may contact the TCR. We found that Ser^5 variants affected activation of T cell effector functions and produced differential levels of effector activity in CTL against the wild-type peptide E75. Modifications that removed the OH group led to variants that induced higher levels of IFN-γ at

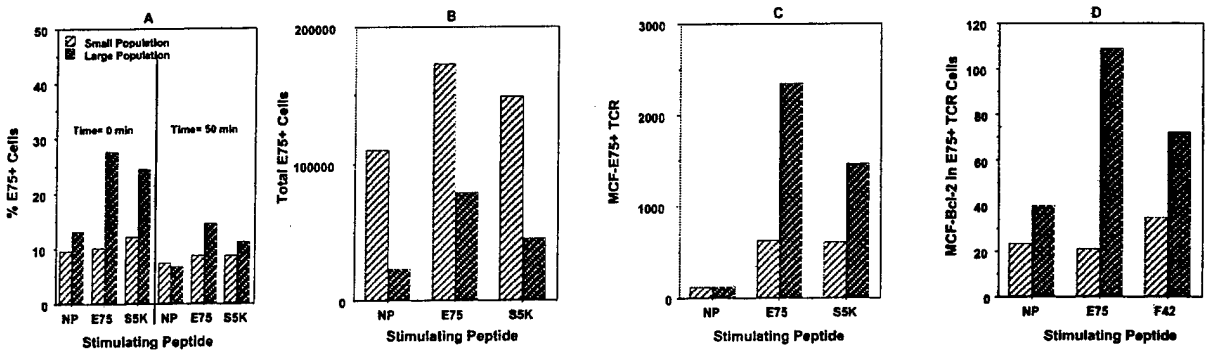

FIGURE 8. Stimulation of S5K-CTL with E75 significantly increased the number of E75$^+$TCR cells. *A*, Percentage of E75$^+$TCR cells in the large (▨) and small (■) lymphocytes was determined immediately after staining and 50 min after washing and incubation of cells in PBS to dissociate low-affinity ($t_{1/2}$ < 50 min) TCR-dE75 complexes. Most small lymphocytes recognized E75 with $t_{1/2}$ of >50 min, while ~50% of large lymphocytes had a $t_{1/2}$ of 50 min for E75. *B*, Increase in the numbers of E75$^+$TCR cells of S5K-CTL after stimulation with E75 and S5K large (▨) and small (■) lymphocytes. The numbers of live cells recovered after stimulation with T2-NP, T2-E75, and T2-S5K, and expansion in IL-2 were 2.7, 3.2, and 2.9 × 10^6 cells, respectively. *C*, Increased levels of expression of E75$^+$TCR in large lymphocytes stimulated with E75 compared with S5K. The differences in MCF in small lymphocytes were minimal: 202 for E75, 180 for S5K. *D*, Increased levels of expression of Bcl-2 in E75$^+$TCR large lymphocytes but not in small lymphocytes at stimulation with E75 or S5K. All determinations were performed in the same experiment. Results are from one determination representative of two with similar results.

priming. In addition, CTL primed by the variant S5A recognized E75 better in lytic assays than did CTL induced by E75. In contrast, modification of E75 by extending its side chain with an aminopropyl group lead to the S5K variant, which induced IFN-γ in two strong responders to E75 but was not a better inducer of E75-CTL-specific activity. In a third donor, weak responder to E75, the potency of E75 and S5K to induce IFN-γ was similar at priming and restimulation. S5K-CTL recognized E75 with lower affinity than E75-CTL. Only at high concentrations of E75, its recognition by E75-CTL and S5K-CTL was similar. Sequential stimulations S5A→S5A, S5G→S5G, and E75→E75 led to death rather than to CTL expansion.

A possible explanation for these effects may be provided if the effects of water (H_2O) molecules are considered. The OH group of the Ser^5 can form H-bonds with residues in TCR. Intercalation of water molecules and formation of H-bonds with the OH group of Ser may decrease the affinity of binding to the TCR, while elimination of OH group may increase the number of hydrophobic interactions. Because the sequence of the TCR and crystal structures of TCR-E75-HLA-A2 complexes are not yet available, we could not define the role of water molecules in the stimulation. Thus, deletion of the OH and CH2-OH (hydroxymethyl) groups induced death by overstimulation. Repeated stimulations with S5K minimized S5K-CTL losses due to apoptosis compared with stimulations with S5A, S5G, and E75. E75 and S5K were similar in their ability to induce IFN-γ. The signal from S5K was weaker than the signal from E75 in that S5K induced significantly lower levels of IL-2 in the S5K-CTL than did E75 (A. Castilleja and C.G. Townsend, unpublished observations).

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Once S5K-CTL were established and were protected from apoptosis by restimulation with S5K, signals from the wild-type E75, or variants with Ala^7 side chain extended with 3CH2 groups, or Phe^8 with side chain shortened with 1CH2 group, induced even higher Bcl-x_L:Bad ratios. In S5K-CTL, E75 also increased the levels of TCR expression and Bcl-2 expression more than S5K. Considering that S5K was recognized with lower affinity than E75 by E75-induced CTL, it is possible that S5K is a weak CTL activator similar to homeostatic inducers (33, 34). A possible explanation for the low affinity of S5K-CTL for E75 is that the stimulus is not sufficiently strong to bring TCR together in the appropriate conformation for wild-type Ag recognition. This may have the advantage of extending the life of such CTL. Further studies with distinct agonists should address this question.

One important consideration now emerging from lymphocyte activation studies is that the CTL response to an Ag first expands and then contracts to bring down the number of activated effectors (35, 36). Reduction in the number of activated CTL is initiated by Ag and manifests by induction of apoptosis at restimulation a phenomenon that is amplified by IL-2 (37). The development of agonistic variants that more strongly activate antitumor effector CTL is a necessary requirement for immunotherapy. Such CTL may be useful if they can mediate immediate effects, i.e., tumor eradication upon activation. Repeated stimulations/vaccinations with strong agonistic variants may lead to depletion of highly activated effectors (38, 39). This raises concerns regarding the use of agonistic variants that are stronger than the nominal Ag in cancer vaccination for induction of central and peripheral memory CTL, because the life span of T cells activated by agonistic variants may be limited. An additional consideration emerging from activation studies is that agonist-induced effectors should survive and maintain their lytic function at encounter with the wild-type tumor Ag. We noted that CTL induced by wild-type E75 showed poor viability after two to three rounds of stimulation. This pattern of response is in agreement with the general pattern of responses to activation by self-specific T cells to avoid induction of autoimmunity (40).

Activation of antitumor effector CTL by weak agonists followed by wild-type Ag is a novel approach to promote their expansion and functional competence that has not been described before in human tumor systems. Similarly, protection from apoptosis and expansion of these cells by the self-peptide tumor Ag is also a novel finding for tumor systems. Such effectors may be useful for controlling the growth of tumors that express high levels of tumor Ag (e.g., HER-2). In addition, low-level activation of effector functions by weak agonists that can also induce homeostatic proliferation may be useful for immunotherapy after chemotherapy or radiation treatments, both of which are known to reduce leukocyte counts. This possibility is supported by studies with experimental models showing that activated low-avidity CTL that are specific for a self Ag can induce tumor rejection (40), and that stimulation of low-affinity clones can break tolerance to T cell epitopes (41, 42). Earlier studies demonstrated that differential TCR signaling

can regulate functional activation and apoptosis in T cells (43). High-strength TCR-Ag interactions lead to activation-induced cell death, while low-strength TCR-Ag interactions can promote death by neglect. However, depending on the nature of TCR-Ag interactions, a range of cellular responses can be induced to avoid cell death (44). Recent manipulations of such responses have involved the use of "null ligands" to attenuate the signaling by strong agonists for high-affinity CTL activation (45), the use of stronger agonists to improve the proliferative capacity of low-avidity CTL (46), and the use of molecular modeling to direct repairs in weak/partial agonists (10).

Priming a CTL response to an immunodominant epitope simultaneously results in priming to variants of the peptide sequence that the individual has not encountered (47, 48). Our previous studies demonstrated that ovarian and breast tumor-associated lymphocyte, which recognized E75, also recognized better variants S5A and S5G (49). This suggested that CTLs that recognized these variants were present in patients. The possible contribution of such clones to the immune response against tumor is still unknown (50–52). Our results show that CTL survival and effector function can be enhanced by sequential stimulation with Ag variants followed by wild-type Ag. This strategy allows the response to be followed or shifted to clones that may be endowed with better survival capacity and can differentiate to peripheral memory cells, or clones with better effector function as needed. Because S5A and S5G are stronger agonists than S5K, while F8Y and F8K appear to be weaker agonists than S5K, it will be important to determine how exposure to these variants can maintain the focus of the CTL response to the wild-type tumor Ag, and which vaccination strategy is more effective in maintaining a response against tumors in patients with persistent disease. Ongoing studies in our laboratory aim to address the effects of sequential stimulation with homeostasis inducers, strong agonists, and weak agonists in developing an antitumor response.

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